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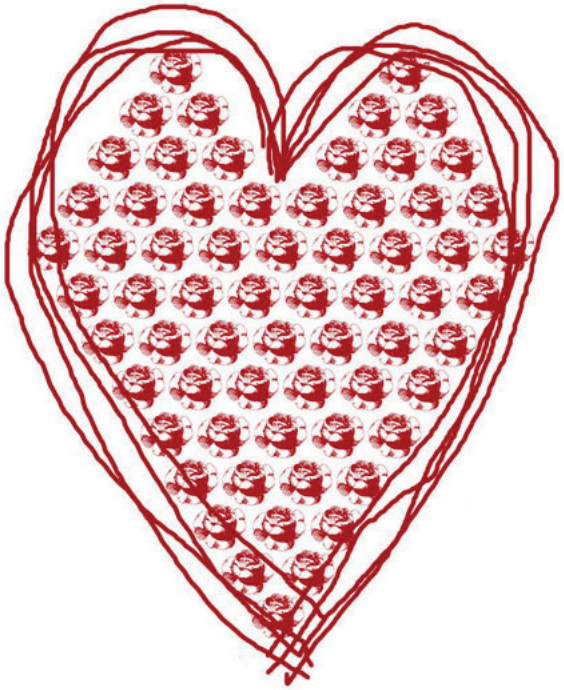
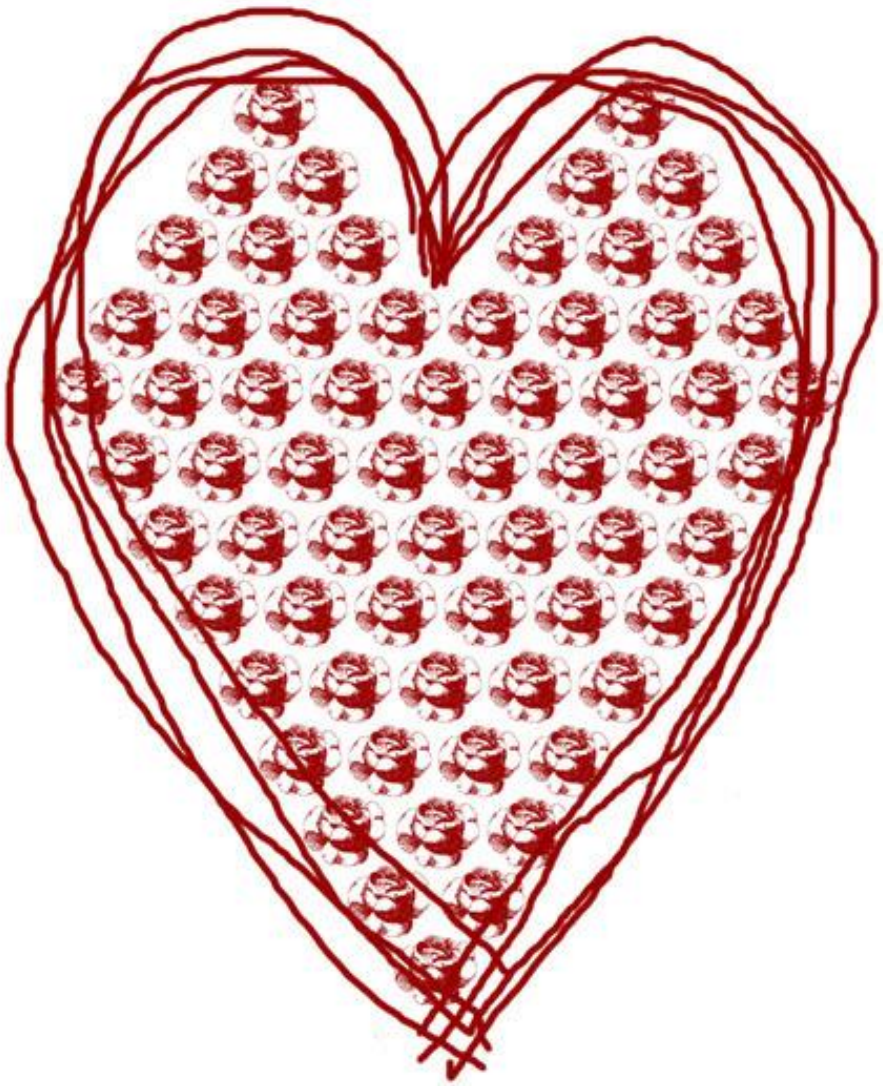


SERS detection of pneumonia in breath of children with cystic fibrosis

Rikke Kragh Lauridsen
PhD Thesis November 2016

DTU Nanotech
Department of Micro- and Nanotechnology

Development of a substrate for surface-enhanced Raman spectroscopy to detect hydrogen cyanide, a biomarker for early *Pseudomonas aeruginosa* lung infection, in the breath of children with cystic fibrosis.



PhD thesis by Rikke Kragh Lauridsen

DTU Nanotech, November 2016.

The cover illustration was reprinted with permission from Michelle Ward, whose sister has cystic fibrosis.

Sixty-five roses is a name often used by CF children for their disease.

Preface

This is not a typical Nanotech PhD. It was not built upon two solid blocks of a BSc and a MSc in physics, but laid on the corners of applied spectroscopy, clinical development, chemistry and microbiology. Instead of the usual three years, it has taken four, because it was done at reduced time, so I could still have time for my family. With educations in food science and technology and my background working in a medical device company, I am grateful that Anja trusted ME to work on this fantastic project.

The work was carried out at Technical University of Denmark, Department of Micro- and Nanotechnology (DTU Nanotech), supervised by Professor Anja Boisen and co-supervised by Researcher Tomas Rindzevicius. Second co-supervisor was Professor Søren Molin from the Novo Nordisk Foundation Centre for Biosustainability, DTU, in collaboration with MD Helle Krogh Johansen from the Department of Clinical Microbiology, Copenhagen University Hospital, Rigshospitalet. Part of the work was carried out at University of Copenhagen (KU), Department of Food Science; at the Danish Paediatric Pulmonary Service of Rigshospitalet; and at DTU, Department of Systems Biology.

Abstract

Cystic fibrosis (CF) is the most frequently inherited disease in the Western world, and also the one with the highest morbidity and mortality. The main reason is chronic lung infections caused by the pathogenic bacterium *Pseudomonas aeruginosa*, which is well-adapted to the thick and dehydrated mucous in the CF airways. Established methods to detect *P. aeruginosa* in young CF children are invasive and lack sensitivity, which is why novel approaches are being investigated. *P. aeruginosa* emits hydrogen cyanide (HCN) gas, which can possibly be used as a biomarker for early *P. aeruginosa* colonisation, if it can be detected in the breath. It was investigated if a nanopillar substrate for surface-enhanced Raman spectroscopy (SERS), developed in the Nanoprobes group, could be optimised for gas phase detection of HCN. The project consisted of 3 steps, of which the first was to establish a chemical method to detect cyanide on the substrate in relevant concentrations, preferably in gas. Step I was split up into two parts; one for HCN detection in the gas phase, and one for detection of potassium cyanide (KCN) in serial dilutions to reach sufficiently low CN concentrations and verify the limit of detection. Once this was done, Step II was to measure HCN(g) from emissions of *P. aeruginosa*; first from the established reference strain, the wild type PAO1. Secondly, it was relevant to study clinical *P. aeruginosa* strains, isolated for the first time from CF children (the wild type-like strains), and then compare to SERS measurements on later strains, isolated from the same patients after their infection became chronic and the *P. aeruginosa* had mutated in the *lasR* gene, which is essential to HCN production. Step III was a clinical trial, where children with CF would blow into a bag containing the SERS substrate, which was then measured, to see if HCN was detected when a new *P. aeruginosa* colonisation occurred; and data was correlated to culturing of sputum from the patient's lungs.

The SERS substrate was optimised, and setups were developed for HCN(g) detection, for SERS detection of HCN from bacterial volatiles, and for collection and SERS substrate exposure to human breath. Five ppm HCN was successfully detected in gas phase, and KCN was detected down to 10^{-6} M. HCN detection was demonstrated from cultures of *P. aeruginosa* wild types, starting from the end of exponential / beginning of stationary growth phase. HCN was also detected from *lasR* mutated clinical *P. aeruginosa* strains isolated from the airways of children with CF, when the mutation was located at the 5' terminal (downstream) of the gene. *P. aeruginosa* isolates with a mutation at the 3' terminal of the *lasR* gene (upstream) did not emit detectable HCN. Application for ethics' committee was submitted and permission granted to conduct a 4 months' clinical pilot study at Rigshospitalet, including 50 CF patients aged 5-17 years and 19 age-matched control subjects. One CF patient had a new *P. aeruginosa* lung colonisation during the trial, and it was probably detected on the SERS substrate, which had an increased HCN signal compared to the patient's other visits. Additional cases of increased SERS cyanide signal were seen in the breath of some of the children, and it was speculated if they could come from prolonged exposure time or from children being exposed to passive smoking at home. The SERS substrate has a background peak in the Raman spectrum, which needs to be addressed, because it is located very close to the cyanide peak.

Sammendrag

Cystic fibrosis (CF) er den hyppigste arvelige sygdom in den Vestlige verden, og også den nedarvede sygdom med den højeste sygelighed og dødelighed. Den vigtigste årsag er kroniske lungeinfektioner med den patogene bakterie *Pseudomonas aeruginosa*, som er tilpasset den fortykkede og dehydrerede slimhinde i luftvejene hos CF patienter. Etablerede metoder til at detektere *P. aeruginosa* hos små børn med CF er invasive og ikke særligt følsomme, og derfor afprøves der nye metoder. *P. aeruginosa* udskiller hydrogen cyanid (HCN) gas, som muligvis kan bruges som biomarkør for tidlig *P. aeruginosa* kolonisation, hvis det kan detekteres i udåndingsluften. Det blev undersøgt, om et "nanogræs" substrat til surface-enhanced Raman spektroskopi (SERS), udviklet i Nanoprobes gruppen, kunne optimeres til gasfase detektion af HCN. Projektet bestod af 3 trin, hvoraf det første var at etablere en kemisk metode til at detektere cyanid på substratet i relevante koncentrationer, fortrinsvis i gas. Trin I blev opdelt i 2 dele; en til HCN detektion i gasfase, og en til detektion af kaliumcyanid (KCN) i fortyndingsrække for at nå ned på tilstrækkeligt lave koncentrationer og verificere detektionsgrænsen. Da det var gjort, gik Trin II ud på at måle HCN(g) fra emissioner af *P. aeruginosa*; først fra den etablerede reference-stamme, vildtypen PAO1. Derefter var det relevant at undersøge kliniske *P. aeruginosa* stammer, isoleret for første gang fra CF børn (vildtype-agtige stammer), og så sammenligne med SERS målinger på senere stammer, isoleret fra de samme patienter, efter deres infektion blev kronisk og *P. aeruginosa* havde muteret på *lasR* genet, som er essentielt for HCN produktion. Trin III var en klinisk afprøvning, hvor børn med CF pustede i en pose med SERS substratet indeni; og det blev efterfølgende målt på, for at se, om HCN blev detekteret, når en ny *P. aeruginosa* kolonisation opstod; og data blev korreleret til dyrkning af sputumprøver fra patientens lunger.

SERS substratet blev optimeret, og opstillinger blev udviklet til SERS detektion af HCN fra gas og bakteriedampe, samt til opsamling af og eksponering for udåndingsluft. Fem ppm HCN blev succesfuldt detekteret i gasfase, og KCN blev detekteret ned til 10^{-6} M. HCN detektion blev demonstreret fra dyrkningskulturer af *P. aeruginosa* vildtyper, fra slutningen af den eksponentielle / begyndelsen af den stationære vækstfase. HCN blev også detekteret fra *lasR* muterede kliniske *P. aeruginosa* stammer isoleret fra luftvejene hos børn med CF, når mutationen var lokaliseret i 5' enden (nedstrøms) af *lasR* genet. *P. aeruginosa* isolater med en mutation i 3' enden af *lasR* genet (opstrøms) udskilte ikke detektérbart HCN. En ansøgning blev sendt til etisk komité, og tilladelse blev givet til at gennemføre et 4 måneders klinisk pilotstudie på Rigshospitalet, med 50 CF patienter i alderen 5-17 år og 19 alders-matchede kontroller. Én CF patient fik en ny *P. aeruginosa* lunge-kolonisation i løbet af afprøvningen, og den blev sandsynligvis detekteret på SERS substratet, som havde et forhøjet HCN signal sammenlignet med patientens øvrige besøg. Flere tilfælde af forhøjet SERS cyanid signal blev set i udåndingsluften fra nogle af børnene, og det blev spekuleret, om det skyldtes forlænget eksponeringstid eller, at nogle af børnene blev udsat for passiv rygning i hjemmet. SERS substratet har en baggrundstop i Raman-spektret, som skal afhjælpes, fordi den ligger meget tæt på cyanidtoppen.

Acknowledgements

Product development is a matter of team work, because you need to take the device from basic research and move it through various stages, where different people can have expertise within each subfield. Therefore you need to trust and involve the surrounding people in order to take the development as far as you can – in the case of medical devices for the ultimate benefit of the patients. Throughout my project I have been lucky to have many such people, who were willing and able to deliver quality input, guidance, help and feed-back, because they trusted in me and wanted to help the patients in this great project.

First of all I am deeply thankful to my manager and main supervisor Anja Boisen, who is the best leader and inspirer I could ever dream of. I admire your integrity and thank you for your trust, support and good advice. I never felt bad coming to you; and if it had not been for you, I would never have been able to deliver this piece of work. If not for Flemming Larsen, the project would not have existed. Thanks for having the idea for it and for mentoring me until it became a PhD project. I also must mention Michael Stenbæk Schmidt, who invented the nanopillar SERS substrate. Thanks for doing so.

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I would like to bring a special thanks to my friend and former supervisor Søren Balling Engelsen, who has helped me throughout this project, too. Thanks for your support and hospitality, for helping me with Raman issues and letting me use your equipment; both when I came for my external visit, and ever since, when I have come by with substrates that had been exposed either in the hospital or in the lab. I have always felt welcome in your group. Kaiyu Wu, you are an extraordinary researcher and colleague! I thank you for taking responsibility for developing the SERS substrate, for sharing your knowledge and numerous times having explained to me what SERS is all about.

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Last, but not least, I am grateful to my Mother who is probably one of the only persons who will actually read this thesis; and to my sisters, Tine and Louise. We have been through some rough times together to learn that we have each other. I cordially thank my husband Bo for always being there, supporting me in every way. And our children; Erik, for showing pride and interest in what I do; and Sofie, for being patient and wise enough to wait until Mama had time to play ☺

Table of Contents

1.1 Background and funding

1.1a *P. aeruginosa* lung infections in cystic fibrosis

Chronic lung infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* are mainly seen in patients with cystic fibrosis (CF), primary cilia dyskinesia (PCD), and chronic obstructive pulmonary disease (COPD) [Gibson *et al*., 2003; Gallego *et al*., 2014]. These patients are infected because they are unable to clear their airways properly, and mucous inhabited by microorganisms is getting stuck [Sommer *et al*., 2016]. *P. aeruginosa* can be found in soil and water reservoirs, where it does not affect healthy individuals, unless they have burn wounds, indwelling catheters or are immunosuppressed [Lorentz *et al*., 2016]. In CF patients chronic *P. aeruginosa* lung infections are the main cause of morbidity and mortality. CF is the most common autosomal recessive genetic disorder, and patients have inherited a defect gene on chromosome #7 from both parents. The gene is called the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and encodes for a protein that regulates salt and water transport across secretory cells in the body, leading to sticky and dehydrated secretions. The most common mutation is a double deletion of phenylalanine in position 508 (ΔF508) of the CFTR protein [Schwartz *et al*., 1990]. Speculations about the background for the success of this particular mutation concern advantages in surviving e.g. cholera and typhoid fever, because the cholera toxin and *Salmonella typhi* need functional host CFTR channels to enter the cells [De Boeck & Ashlock, 2011].

Because *P. aeruginosa* airway colonisation can be difficult to discover in the initial stages and need to be treated with antibiotics before they become chronic [Marvig *et al*., 2015], new methods are being investigated. *P. aeruginosa* emits the poisonous gas hydrogen cyanide (HCN), which can be an advantage in killing competitive flora in the surroundings. In this project a nanostructured surface-enhanced Raman spectroscopy (SERS) substrate, developed for detection of explosives in the air [Schmidt *et al*., 2012], is optimised for detection of the *P. aeruginosa* biomarker HCN in the breath of children with CF. The triple bond between C and N in cyanide is utilised, because triple bonds give rise to a distinct peak in a Raman spectrum. The original SERS substrate is covered with silver (Ag), and the approach in this PhD project will be instead to use gold (Au) on top of the nanopillar surface, because of cyanide's high affinity to Au [Murray & Bodoff, 1986; Cho *et al.*, 2002]. At some point, *P. aeruginosa* starts to adapt to the environment in the airways, and a mutation in the *lasR* gene can mean that it does not produce HCN anymore [Pessi & Haas, 2000].

1.1b Raman spectroscopy

Spectroscopy concerns the interaction between light and matter. A beam is directed towards the sample, and light is collected after their interaction, providing information about the chemical / physical nature of the sample. Raman spectroscopy investigates the inelastically scattered part of the light, i.e. the light scattered from the sample that has changed vibrational energy during the interaction. It was illustrated in 1928 by Raman and Krishnan, who let sunlight through a telescope lens followed by two complementary filters. After the complementary filters they placed a sample, and no light could be seen at an angle to the sample, because it had all been filtered out. Then they moved the yellow-green filter and placed it after the light's passage through the sample and now light could be seen. Due to the blue-violet filter, only yellow-

green light was let to the sample, and it could be expected to be filtered out by the yellow-green filter after the sample; but the light's interaction with the sample had changed its wavelength, which is why scattered light from the sample could be seen after the yellow-green filter. The spectrum gives Raman intensity as a function of exchanged energy, and since strong bonds vibrate at high frequencies, the triple bond between C and N in cyanide gives rise to a distinct peak at 2135 cm^{-1} in the triple bond / cumulated double bond region between 2000-2200 cm^{-1}, which is normally flat [Raman & Krishnan, 1928; Smith & Dent, 2005].

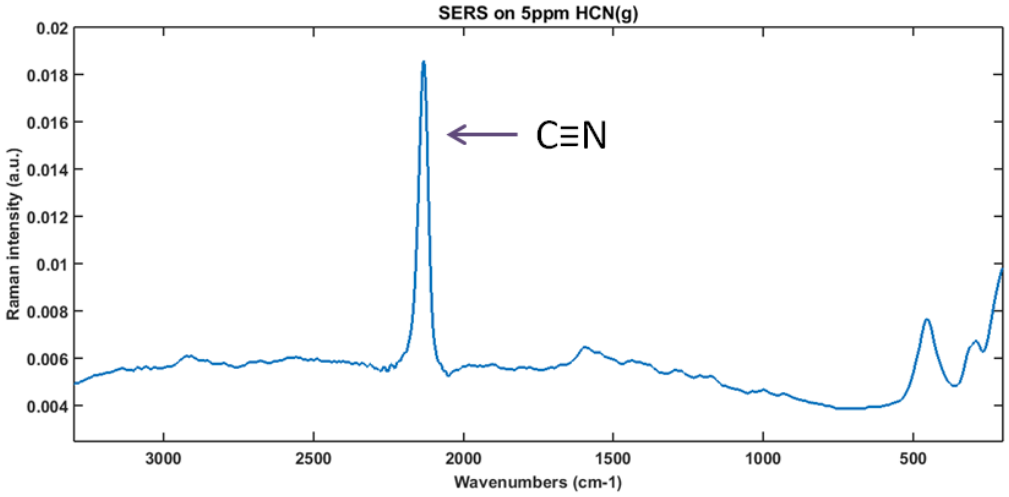

Figure 1.1: Surface-enhanced Raman spectrum (SERS) of 5 ppm HCN(g). The triple bond peak is marked by an arrow.

Because only a small fraction of the scattered light is scattered inelastically (~10^{-8}), there is a need for enhancement of the Raman signal. We have optimized the signal by the use of surface-enhancement by a nanostructured silicon (Si) surface covered with Au. Even if the region is supposed to be flat, there is, however, a background peak on the Au substrate at 2127 cm^{-1}, which is very close to the C≡N stretching peak, sometimes confounding the results. The background peak has also been reported elsewhere [Premasiri, *et al*., 2001].

1.1c Medical device development

The aim of the project was to develop a medical device able to detect ppb HCN from human breath. Devices cannot be built on test systems, theories and assumptions alone. Medical devices must be taken to clinical trials. To communicate with the end users and watch them interact with the device you are developing, can give valuable information that had not been thought about before. According to The Lean Startup, developers must build a minimum viable product and put it into the "Build – Measure – Learn" feedback loop to start learning as quickly as possible. The prototype does not have to be perfect, it must be built on a minimal amount of development time and effort and be able to provide valuable feedback from those who will be using the product in the future [Ries, 2011]. With a background in the medical device industry I know that there is a certain scope of a project (e.g. in terms of time and money), and you must prioritise and plan accordingly in order to be able to constantly move closer to the goal.

1.1d This project

The project indeed is multidisciplinary, dealing with product development, chemistry, physics, Raman and SERS spectroscopy, cleanroom fabrication, physiology, microbiology, genetics, clinical trial management as well as logistics. One person cannot be an expert of all, and I have tried to prioritise the insights necessary for the project to keep moving forward, combining the fields as well as I could. In case there are terms, which are unfamiliar to the reader, lists of expressions can be found at the beginning of each theory section in Chapter 2. The PhD project is part of the Sapere Aude project "NAPLAS", which is developing in-depth understanding of the optical properties and plasmon coupling effects in the nanopillar SERS substrate and building miniaturized, label-free sensors for (semi-)quantitative detection of toxic molecules like PCB and HCN. It was funded by The Danish Council for Independent Research.

1.2 Motivation

In order to detect microorganisms as early as possible and thereby avoid a chronic infection, samples must be collected regularly from the airways of CF patients. Today the gentlest way to obtain a microbiological sample from the lungs is by spontaneous sputum production, also called *expectoration*. The patient coughs up a sputum sample from the lungs, which is then cultured to detect microbes. Children below the age of 8-9 years do not produce sufficient amounts of sputum to expectorate, and therefore other methods must be taken into use in order to find out whether the child has a bacterial lung infection and needs treatment before it settles and causes a chronic infection. In increasing order of invasiveness comes; *pharyngeal swaps*, where a wooden stick holds down the tongue while a nylon or cotton stick is inserted through the mouth to the throat to catch what might be colonising the pharynx and surroundings; *induced sputum*: hypertonic saline is sprayed into the airways, causing irritation and inducing coughing, whereby a sputum sample is obtained, and there is a high risk of getting the environment contaminated; *endo-laryngeal suction*: a plastic catheter is inserted through the mouth or nose and into the throat down to the larynx, provoking a cough reflex, and a sputum sample is obtained through the tube; *broncho-alveolar lavage* (BAL), which is performed under general anaesthesia, where part of the lung is rinsed with sterile saline, and subsequently the fluid is cultured.

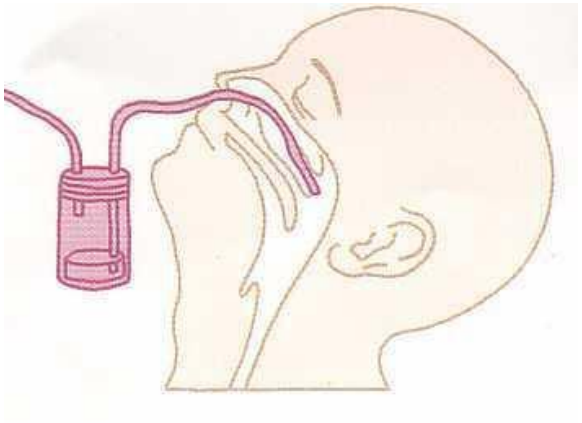

Figure 1.2: Principle of endo-laryngeal suction, which is used in the CF clinic at Rigshospitalet to obtain sputum samples from non-expectorating children [www.nhspathology.fph.nhs.uk].

After a while in the lungs, the body starts to produce signs of persistent bacterial colonisation or infection, in terms of e.g. antibodies against *P. aeruginosa*, which can be detected from a blood sample. The motivation is therefore, that children with CF need a more sensitive and less invasive method to detect early *P. aeruginosa* airway colonisation.

1.3 Review of existing research

Due to the unpleasant and highly invasive methods to discover *P. aeruginosa* in the lungs of CF children unable to produce sputum, breath analysis may offer a more acceptable, and perhaps even more sensitive, way to detect this pathogen. The first step is to discover which specific biomarkers could be used. Secondly, moving closer to point-of-care diagnostics, it is relevant to focus on detection in the breath, followed by Raman and SERS applications in healthcare and in the detection of HCN.

1.3a Probing of *P. aeruginosa* biomarkers

Many things have happened since Castric's interesting studies on HCN production by *P. aeruginosa* in 1975. His method was to catch the cyanide headspace onto an alkaline filter strip trap, and for confirmation he acidified both the culture and the evolved gas with H_2SO_4, passed it through $AgNO_3$ / HNO_3 and filtered the precipitate to a white solid, presumably AgCN, which was added to 10N H_2SO_4 and heated to 100°C, again trapping the gas in base. The trapped material tested positive for cyanide in picrate, Aldridge, and Prussian Blue tests [Castric, 1975]. The results are nice, but a simpler and more pleasant test would be preferable to verify that HCN is in the culture headspace – or in the breath of a child infected with *P. aeruginosa* in the lungs. Within the last decade several methodologies have been applied, most of which dealing with mass spectroscopy (MS) or cyanide ion selective electrodes. In 2005, Carroll and coworkers used selected ion flow tube mass spectroscopy (SIFT-MS) to detect HCN and other volatiles in the headspace of *P. aeruginosa* cultures, suggesting that HCN could act as a specific *P. aeruginosa* biomarker in the breath of infected patients [Carroll *et al*., 2005].

Pedersen *et al*. suggested using immunoproteomics from a blood sample as a means of detecting *P. aeruginosa*. They used circulating antibodies in plasma from CF patients, which were screened against a 2D protein macroarray of a *P. aeruginosa* protein extract. Antigenic *Pseudomonas* PAO1 proteins were then captured by the plasma antibodies and arrayed by 2D gel electrophoresis, from where protein spots were proteolytically digested, so the purified peptides could be analysed by MALDI-TOF MS against various databases for identification of immunoreactive profiles [Pedersen *et al*., 2005]. In 2008, Ryall measured CN^- in sputum of CF- and non-CF bronchiectasis patients with a *P. aeruginosa* airway infection, using an ion-selective electrode. They demonstrated for the first time that cyanide could be detected in sputum, with levels in sputum from CF patients being higher (up to 130 µM) than in bronchiectasis patients (72 µM), as compared to control samples free of *P. aeruginosa*, where no cyanide was detected. They also mentioned that in some of the *P. aeruginosa* positive sputum samples no cyanide was detected [Ryall *et al*., 2008].

In 2011 Stutz and coworkers stated that cyanide in BAL samples from CF children was not diagnostic for *P. aeruginosa*, because applying a cyanide ion selective electrode, cyanide was found in BAL samples, which cultured both positive and negative for *P. aeruginosa*. They also said that BAL culture was not a very sensitive technique, and that the cyanide found could be attributed to inflammatory disease [Stutz *et al*.,

2011]. To test a new method, for the outcome to be valid, it is important that the method you are testing against is reliable. If the authors are unsure of the sensitivity using BAL, they must be careful not to reject a method which has the potential to be even more sensitive. The study by Ryall and coworkers was followed up in 2011 by Savelev *et al*. who measured volatile organic compounds in the headspace of sputum samples from *P. aeruginosa*-infected CF- and non-CF bronchiectasis patients, using solid phase micro-extraction MS. They concluded that 2-nonanone could be an additional *P. aeruginosa* biomarker [Savelev *et al*., 2011], which was rejected in 2012 by Jünger *et al*. who found it in the headspace of several other pathogens, including *Eschericia coli*, *Klebsiella pneumoniae, Enterobacter cloacae* and *Serratia marcescens*, as determined by thermal desorption GC-MS [Jünger *et al*., 2012]. According to Bos *et al*., 2-nonanone is also produced by *Staphylococcus aureus*, leaving it as a poor biomarker for *P. aeruginosa* [Bos *et al*., 2013]. Hydrogen cyanide thus seems to be the most promising *P. aeruginosa* biomarker. But with a molar mass of 27.03 g/mol, it is difficult to establish a GC-MS technique for the determination of HCN concentrations, since most GC-MS systems cannot go below 30 g/mol. Shestivska *et al*. therefore suggested using methyl thiocyanate (CH_3SCN) as a possible *P. aeruginosa* biomarker instead [Shestivska *et al*., 2012].

1.3b *P. aeruginosa* in breath

In an attempt to diagnose *P. aeruginosa* noninvasively, Vogelberg and coworkers tried to detect bacterial DNA using PCR on breath condensates from 13 young CF patients known to have *P. aeruginosa* or *Burkholderia cepacia* in their airways. As reference, sputum samples were acquired whenever possible, but only the sputum samples and none of the 32 breath condensates contained any bacterial DNA [Vogelberg *et al*., 2003]. This is interesting, because some *P. aeruginosa* lineages are known to be able to spread among CF patients [Marvig *et al*., 2013], and this could be expected to occur via breath aerosols. Compared to sputum culturing, the use of PCR may be a step forward, when it comes to sensitivity. Unfortunately it is more expensive, and the method still needs sputum sampling. Using GC-MS, Scott-Thomas *et al*. in 2010 detected higher amounts of 2-aminoacetophenone (2-AA) in the breath of CF patients colonised with *P. aeruginosa* than in the control groups and suggested 2-AA as a possible biomarker for *P. aeruginosa* [Scott-Thomas *et al*., 2010]. The year after they found that 2-AA levels in breath can be confounded by food intake such as corn products, canned tuna, egg white and beer [Scott-Thomas *et al*., 2011].

In 2013, Zhu *et al*. published a study on secondary electrospray ionization mass spectroscopy (SESI-MS) on the breath of ventilated mice with a *P. aeruginosa* or *S. aureus* lung infection. Applying partial least-squares regression in combination with discriminant analysis (PLS-DA) to the SESI-MS breathprints they were able to completely separate *P. aeruginosa*-infected mice from *S. aureus*-infected mice and from a third group of control mice. They even said it might be possible at an early infection stage to identify which mice were able to clear the infection by themselves and which would need antibiotics. In conclusion, instead of looking for single biomarkers, they recommended to use the entire breathprint in the analysis to discriminate between the groups [Zhu *et al*., 2013a]. Dummer *et al*. used SIFT-MS to quantify HCN in the breath of 6 healthy, non-smoking volunteers with a mean age of 35 years, comparing nose- and mouth-exhaled breath. Due to elevated HCN in mouth-exhaled samples, they concluded that breath samples should be exhaled via the nose. Twenty-six CF and non-CF bronchiectasis patients with a mean age of 62 years delivered nose-exhaled samples and no significant relationship between *P. aeruginosa* status and end exhaled HCN concentrations was found, so they concluded that HCN cannot work as a *P. aeruginosa* biomarker [Dummer *et al*., 2013]. The fact that the patients included in this study had a relatively high age

suggests that many of them possible already had a chronic infection, which again means that their *P. aeruginosa* can have mutated into *lasR* defect strains, which had ceased to emit HCN. This was the experience from the *in vitro* studies presented in Paper II.

Gilchrist and coworkers investigated nose- and mouth-exhaled breath from 10 adult chronically *P. aeruginosa*-infected CF patients and compared to 10 adult CF patients without *P. aeruginosa*, using SIFT-MS. They detected acetone, ethanol and HCN. Acetone content was used as a measure of consistent sampling rate, whereas ethanol is generated in the mouth by enzyme activity of bacteria and saliva. Nearly all chronically infected subjects had elevated HCN levels in their mouth-exhaled breath, whereas four of their nose-exhaled samples did not. Nevertheless, nose-exhaled breath samples of chronically infected patients had significantly higher HCN concentrations than those free from *P. aeruginosa*; and mouth-exhaled breath showed the same trend, although some of the HCN was expected to come from bacterial activity in the mouth [Gilchrist *et al*., 2013]. Finally, in 2015 they published a large study on HCN concentrations in the breath of 233 children with CF, again using SIFT-MS, but on mouth-exhaled breath, collected over two years from 8 centres in UK (The SPACE study). Findings were that half of the 57 children with newly acquired *P. aeruginosa* infections had elevated HCN in their breath, some up to 2 visits prior to positive culture, whereas the other half did not. This other half had HCN levels in their breath similar to those who remained free from *P. aeruginosa*. Additionally, there were no significant differences between the two groups regarding age or *P. aeruginosa* status prior to being colonised [Gilchrist *et al*., 2015]. Like the study by Dummer *et al*. the authors included chronically infected patients; who, with the experience gained from this PhD, should probably not have been included in the study. They had not had any positive culture for the past 12 months prior to inclusion, which can question the reliability of the swab test used, also during the trial [Bonestroo *et al*., 2010]. To avoid the inconveniences of the presented methods pointed out in Papers I and II, our approach was to detect HCN using a Au SERS substrate for cyanide adsorption, measured with Raman instrumentation, which has the potential to be down-scaled for home or point-of-care diagnostics at an affordable cost.

1.3c Raman / SERS for diagnostics

Due to the method's novelty, the literature on Raman / SERS methodology on exhaled breath is sparse. A recent study on a complex gas mixture was published by Jürgen Popp's group, who used a microstructured hollow core photonic crystal fibre (HCPCF) for fiber-enhanced Raman spectroscopy (FERS), simultaneously detecting H_2, CO_2, N_2O, O_2, CH_4, and N_2 at ppm levels, combining rotational and ro-vibrational FERS. In the colon, *E. coli* generates high levels of H_2 in lactose intolerance and fructose malabsorption, and the goal was to apply the method for hydrogen breath testing of these malabsorption disorders [Hanf *et al*., 2015]. The problem with the application of the presented method is its ppm level detection, which for our intended use needs to be ppb [Enderby *et al*., 2009].

The first attempt to detect bacteria using SERS was probably done in 1998 by Efrima and Bronk, who used Ag colloids to enhance the Raman spectrum of *E. coli*. The bacteria were soaked in $NaBH_4$, centrifuged and resuspended in $AgNO_3$, producing a rough film on the outside of the bacterial wall. The resulting SERS spectrum was rich in bands that could be attributed to peptidoglycans of the cell wall [Efrima & Bronk, 1998]. In order to be used for non-invasive detection of *P. aeruginosa*, the approach of course is too rough, and VOC biomarker detection in comparison would be an advantage. In 2007, Alexander and Le used the commercially available Klarite™ substrate to identify four different *Bacillus* spore suspensions deposited

onto the substrate and dried at room temperature. Due to unique features in each of the spores' spectra, they could be classified univariately, i.e. based on single Raman bands. For a more general identification of bacterial spores the authors recommend using multivariate tools on the entire SERS spectrum, to predict or classify each type of spore [Alexander & Le, 2007]. The Klarite^{TM} substrate was also tested in the present PhD project. Although high uniformity, it suffered from poor enhancement, and the substrate is not on the market anymore.

There exists some good review papers on Raman applications [Das & Agrawal, 2011]; SERS and SERRS applications [McNay, *et al*., 2011]; medical SERS applications [Xie & Schlücker, 2013]; and potential SERS applications for disease detection and treatment [McAughtrie, 2014]. Of special interest are some papers from van Duyne's group, who reported SERS quantification of glucose down to micromolar levels using a silver film over nanosphere (AgFON) surface with a partitioning self-assembling monolayer (SAM) of 1-decanethiol to preconcentrate the glucose close to the Ag surface, which was also protected against oxidation by the SAM. Partial least-squares regression, using full cross validation (leave-one-out) was used to validate the prediction of glucose from 0-250 mM and 0-25 mM [Shafer-Peltier, 2003]. Using full cross validation is probably not the best way to validate a model's prediction error, because by leaving one sample out at a time, and then including it in the next iteration can result in over-optimistic prediction errors, and it is probably not what the authors would have applied today [Esbensen *et al*., 1997]. Later they published new results using AgFON SERS, this time without the SAM, detecting the *Bacillus subtilis* spore biomarker calcium dipicolinate to a limit of detection of 2.6×10^3 spores, as a substitute for the pathogenic *B. anthracis*. This time the results were self-explanatory and did not need multivariate analysis to become evident [Zhang *et al*., 2005]. If anthrax spores thus can be detected by means of a fairly simple method, much could be gained against microbiological warfare. On top they reported on shelf lives up to 40 days for this Ag substrate. This is impressive and can be compared to the tests on our Au substrate, which lasted for at least 17 days, but less than 2 months, referring to section 3.2 on SERS substrate optimisation.

Wu and Cunningham presented a Au SERS sensor able to reversely bind 10 pharmaceutical compounds for continuous real-time monitoring of administered drugs in an intravenous tube. Two sedative or pain drugs in combination could be monitored simultaneously on the flexible polyester substrate inside the flow cell used for the experiments. The assay could be used to monitor drug delivery and prevent overdose, which is of tremendous importance to the wellbeing of the patient [Wu & Cunningham, 2014].

Presented are different substrates, which have been developed for different purposes. *Trans*-1,2-bis(4-pyridol)ethylene (BPE) is a very Raman-active, two-ring molecule with 5 distinct peaks in the region 1000-1700 cm^{-1}, which is often used to benchmark SERS substrates [Kim *et al*., 2010]. But to develop substrates solely based on the comparison of their BPE signal intensities can be risky, unless the intended application for the substrate is BPE detection. At one point I tried a different SERS substrate because, unlike the other Au substrates, this did not have the annoying background peak at 2127 cm^{-1}. But when exposed to HCN, the triple bond peak was completely absent, because the substrate did not enhance any Raman scattering in the region 2000-2200 cm^{-1}, although it was excellent for the enhancement of BPE. In section 3.2, attempts are described to optimise the SERS substrate for the specific application of HCN detection at ppb level in the gas phase.

Chapter 1: Introduction

The PhD project was divided in 3 steps, of which the first was to detect HCN in the gas phase and to demonstrate ppb level detection of cyanide (Paper I). The second step was to detect HCN from emissions of 37 °C *P. aeruginosa* cultures isolated from paediatric CF patients, to mimic detection in the breath (Paper II). The last step was to perform a clinical trial, including children with CF who exhaled into bags with the SERS substrate mounted inside, to verify whether it was possible to detect HCN in the breath of children at an early stage of *P. aeruginosa* airway infection (Paper III). The Experimental part is described in chapter 3 and the papers included in the thesis.

List of expressions – cystic fibrosis

Allele Variants of a chromosome or gene.

Autosome Chromosome which is not a sex chromosome.

BAL Broncho-alveolar lavage; airway rinsing with sterile saline under general anaesthesia.

Bronchiectasis Abnormal widening of parts of the lung, e.g. the bronchial tubes, accompanied by coughing; excess mucus production and coughing up blood.

Colonisation Initial inhabitation by a pathogen without antibody production or inflammatory reaction, as opposed to a chronic infection with an antibody response.

ΔF508 Deletion of a phenylalanine in position 508 on the *CFTR* gene; the most common mutation causing cystic fibrosis.

DNA Deoxyribonucleic acid is made of pairs of chromosomes, which are coiled strands of biopolymers consisting of the four nucleotides; cytosine (C); guanine (G); adenine (A); and thymine (T), arranged in base pairs of C and G, respectively A and T.

Expectorate Sample coughed up from the lungs for microbiological investigation.

Fibrosis Scar tissue in the body.

Gene One gene consists of 1000 nucleotides = 333 amino acids.

Islets of Langerhans Part of pancreas where insulin is produced.

Mucociliary clearance Movement of the small hair-like structures covering airway mucosa to wipe off bacteria and particles.

Neutrophils White blood cells, involved in the immune response.

Osteopenia Reduced bone density; precursor of osteoporosis.

PCR Polymerase chain reaction is used to produce numerous copies of DNA present in a sample.

Recessive Non-dominant allele of a gene, meaning that if both parents are carriers, then the offspring will not necessarily be a carrier or affected by the trait.

Vas deferens Sperm leading duct in the male anatomy.

2.1 Cystic fibrosis

Cystic fibrosis (CF) is a genetically inherited autosomal recessive disease affecting the exocrine secretory glands of the patient. With about 100.000 patients Worldwide it is the most frequently inherited disease in the Western world, and also the one with the highest morbidity and mortality. CF is inherited from both parents who are carrying the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene defect. It is estimated that about 1 out of 34 Caucasians are carriers, and in the "autosomal recessive" term lies that the chances of two carriers getting a CF child is 1:4, while 2:4 will be healthy carriers, and 1:4 will not carry any affected genes [Schwartz *et al*., 1990]. The term "cystic" refers to the cysts developing in the pancreas, and "fibrosis" to the fibroses seen in the pancreas and lungs of the patient.

2.1a Genetics and the CFTR protein

Since CF is a genetic disease, a short introduction to genetics can be useful. Genes are made of DNA strands carrying information about how genetic traits are passed on to the next generation. They are found inside the nucleus of living cells. DNA consists of pairs of chromosomes, which are built of (base) pairs of the nucleotides cytosine (C), guanine (G), adenine (A), and thymine (T). A nucleotide triplet is called a codon and codes for the expression of one amino acid, thus genes can code for proteins. Humans have 23 pairs of chromosomes, of which one is the sex chromosomes. The rest are called autosomes. On the long arm of chromosome #7, the *CFTR* gene is present. About 2/3 of all CF patients have the ΔF508 mutation, which is a deletion of 3 nucleotides coding for the amino acid phenylalanine in position 508 of the CFTR protein [Johansen *et al*., 1991]. The CFTR protein consists of 1480 amino acids. It is a chloride ion channel functioning across secretory cells, and when impaired, it will not fold properly, or may not even reach the cell wall, and a salt imbalance occurs due to altered Cl^- and water exchange across the cell membrane. Secretory cells are found in glands throughout the human body, including sweat glands and mucus glands in the intestinal system, airways and vas deferens.

2.1b Clinical manifestations of CF

The list of CF manifestations is long, and in the following the most common complications are mentioned. The first symptom the parent of a CF child may discover is the salty tasting skin. In the lining of the sweat duct, salt is not being reabsorbed as it should, so an excess of Na^+ and Cl^- ions are led to the skin where water evaporates, leaving the skin salty [Quinton, 2007]. Due to blocked pancreatic and biliary ducts, the lack of digestive enzymes leads to malabsorption of proteins and lipids, and along with thick and sticky intestinal mucosa, this is accompanied by abdominal pain, intestinal blockage (meconium ileus) and fatty, frequent stools. Because vitamins A, D, E and K are fat soluble, these will also be malabsorbed, and the child fails to thrive [Folkesson *et al*., 2012]. Once the nutritional losses have been taken care of, the most common symptoms come from the airways, where coughing and frequent infections of both the sinuses and lungs are seen. The mucociliary clearance is hindered by thick and dehydrated mucus obstructing ciliary movement. Excessive absorption of sodium can occur, further enhancing water reabsorption by osmosis and complicating airway irritation. The build-up of nutritional enzymes inside the pancreas can cause auto-degradation of the islets of Langerhans, leading to insulin deficiency and diabetes. Approximately 13% of Danish CF patients aged 11-16 years have diabetes [Knudsen *et al*., 2012].

When the airways are not cleared properly, the immune system recruits an inflammatory response, attracting neutrophils (white blood cells) to the site. In order to break down intruding cells, the neutrophils release proteases, including elastase, which over time destructs the elastic tissue of the lungs. Already from very early in life the neutrophilic inflammation can be persistent, even when no pathogens are cultured [Sagel, 2003; Gifford & Chalmers, 2014; Chmiel *et al*., 2013; Elizur *et al*., 2008; Heijerman, 2005].

2.1c Living with CF

The diet of a CF patient must be high in salt and calories. For digestion, synthetic enzymes and vitamin supplements must be taken along with meals. Since CF children do not often die from malabsorption, the symptoms from the airways are the dominant signs. Sinus infections are hard to treat, because the bacteria grow in microcolonies, where they are difficult to target with antibiotics [Koch & Høiby, 1993]. Sinus surgery, removing some of the obstructive tissue and polyps followed by cleansing of the sinuses, has proven to be very efficient; also in reducing future lung colonisations [Johansen *et al*., 2012]. Postoperative it is important that the patient keeps the sinuses clear by daily rinsing with saline or perhaps antibiotics [Alanin *et al*., 2016]. To overcome the lack of mucociliary clearance, lung physiotherapy using a PEP mask can be life-extending. Some patients use a vest for chest wall oscillation therapy for ½ hour per day, to loosen excess mucus. If the inflammation and thereby the degradation of the lung tissue is too severe and the lung function is declining markedly, a lung transplant can be necessary.

In order to postpone the onset of chronic lung infections, each country has a certain programme for obtaining sputum samples if the patient is unable to expectorate [Döring *et al*., 2000; Döring & Høiby, 2004]. In Denmark the child attends the CF outpatient clinic once a month, where naso-laryngeal suction is performed. In very young patients, BAL can be necessary, rinsing the lung to culture what is found in the fluid. Sometimes it is also necessary to take a blood sample to monitor the levels of antibodies against Gram-negative bacteria infecting the lungs. Once a colonisation is discovered, antibiotic treatment can start, including inhalations and pills to swallow. If an infection is severe, intra-venous (IV) treatment under hospital admission can be necessary. Danish CF patients with a chronic lung infection spend 2 weeks every 3 months receiving IV antibiotic treatment [Koch & Høiby, 2000]. Different criteria have been applied to define when an infection is regarded as chronic. According to the Copenhagen criteria, six consecutive, monthly sputum samples should be cultured positive, or the blood level of precipitating antibodies against *P. aeruginosa* should be ≥ 2 [Høiby *et al*., 1977]. The Leeds criteria define a chronic infection as when > 50% of ≥ 4 collected samples through the past 12 months have been cultured positive [Lee *et al*., 2003]. In 2008, approximately 80% of the Danish CF patients > 25 years suffered from chronic *P. aeruginosa* airway infection [Hansen *et al*., 2008].

List of expressions – *Pseudomonas aeruginosa*

Cyanogenesis	Cyanide formation
E. coli	*Escherichia coli*, Gram-negative rod, found as part of the normal flora in the colon.
Genome	Complete set of DNA in an organism.
lasR	Gene coding for the LasR protein, regulating cyanogenesis in *P. aeruginosa*.
LB	Luria Broth growth medium
Lysis	Cell death
Mucoid	Biofilm-forming, e.g. a mucoid *P. aeruginosa* forms “slimy” and shining colonies due to mucoid exopolysaccharide (alginate).
OD	Optical density, absorbance as a measure of cell density.
ON	Overnight
Pathogen	Microorganism causing disease in e.g. plants or humans.
QS	Quorum sensing; bacteria working as a community, e.g. causing diseases.
SOP	Standard operational procedure
VOC	Volatile compounds
WT	Wild type organism, as it exists in nature.

2.2 *Pseudomonas aeruginosa*

2.2a *P. aeruginosa* at a glance

P. aeruginosa is a ubiquitous environmental bacterium found in soil and water reservoirs and on aquatic surfaces. It is a Gram-negative, rod-shaped bacterium; which, unlike human cells, does not have a defined nucleus or other membrane-bound organelles. Its DNA can be located all over the cytoplasm, which defines bacteria to the class of prokaryotes. It has a highly advanced chemosensing system and can move towards attractants by flagellar movement [Stover *et al.*, 2000]. Gram-negative bacteria have an outer bacterial membrane, offering better protection against antibiotics than Gram-positives, which only have a thick layer of peptidoglycan in the cell wall. The peptidoglycan layer stains Gram-positive bacteria purple, using Gram-staining, whereas Gram-negative bacteria fail to stay purple after washing, but instead turn red or pink when a counterstain is added.

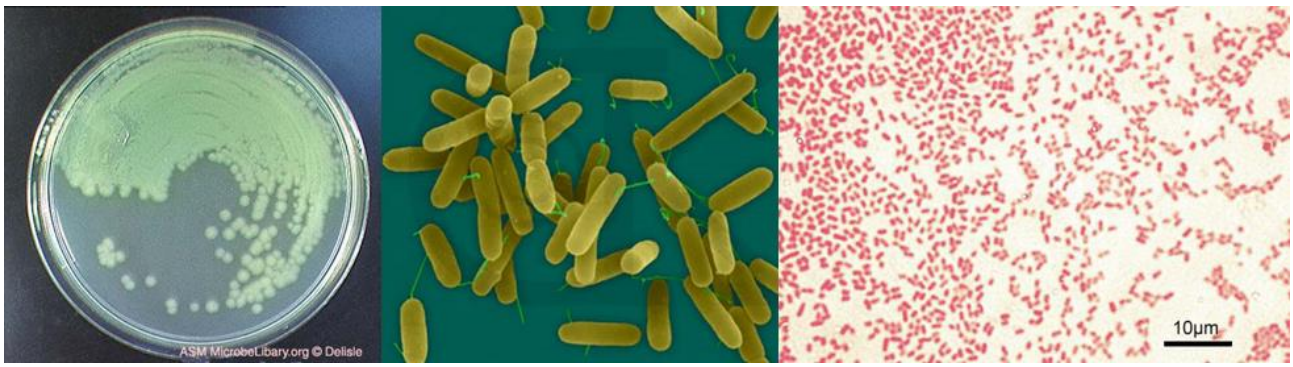

Figure 2.1: *P. aeruginosa*; grown in the lab (Left); as often presented (Middle) Both: [*Pseudomonas*.com]; Gram-staining, seen in microscope (Right) [https://commons.wikimedia.org/w/index.php?curid=49535].

This evolutionary highly adapted pathogen has a large genome size of 6.3 million base pairs (Mbp), as compared to e.g. *E. coli* with 4.6 Mbp. In *P. aeruginosa*, an estimated 8.4% of the genome is involved in regulation, which allows the bacterium to respond to environmental changes and to control virulence, causing damage to the host. In addition to toxins, lipases and proteases are secreted, which can possibly modify or degrade antibiotics administered to the infected host [Stover *et al.*, 2000]. Biofilm production is another challenge, because this slime consisting of DNA, proteins and polysaccharides, enables the bacteria to adhere to surfaces and to stick to each other, hiding from antibiotics [Rasamiravaka *et al.*, 2015]. All these activities are energy consuming, and *P. aeruginosa* has been found to possess about 200 cytoplasmic membrane transport systems for the uptake of nutrients and other molecules [Stover *et al.*, 2000].

2.2b The *lasR* gene and cyanide production

In addition to the inflammatory response of the body, *P. aeruginosa* also produces toxic compounds, which can damage the host. The best known toxin excreted from *P. aeruginosa* is Exotoxin A, which has a mode of action similar to diphtheria toxin, by binding irreversibly to the cell, which then loses the ability to synthesise protein [Brock *et al*., 1994]. *P. aeruginosa* is the only bacterium of relevance to CF airway infections that produces the toxic gas hydrogen cyanide (HCN). It has not been reported to occur in concentrations which themselves can cause damage to humans, but it can be advantageous in the fight against other microorganisms in the lungs. It is synthesised as a secondary metabolite from glycine, probably to regulate the levels of this enzyme, which could otherwise cause lysis of the bacterial cell [Castric, 1975]. It is regulated by the Quorum-Sensing (QS) regulators LasR and RhlR, activating the *hcnA* promoter to start transcription of HCN synthase. The *lasR* gene codes for the expression of the transcriptional activator protein LasR, which is the only regulator that is absolutely required for cyanogenesis [Pessi & Haas, 2000]. Several pathways have been suggested for the formation of HCN from glycine, of which Wissing proposed the glycine dehydrogenase pathway under formation of CO_2:

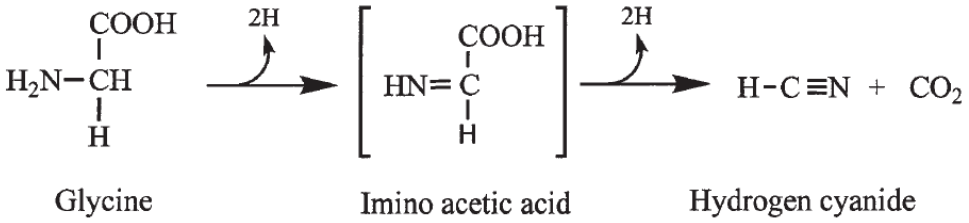

Figure 2.2: Action of HCN synthase or glycine dehydrogenase in the formation of HCN from glycine [Wissing, 1974].

List of expressions – Raman spectroscopy

CCD — A **charge-coupled device** is a device converting incoming photons into electron charges and subsequently digital values.

σ — Spectroscopic **cross section** refers to an effective area that quantifies the probability of a spectroscopic event to occur, when an incident beam hits the target particles. It is defined as the attenuation coefficient (cm^{-1}) divided by the density of the target particles (cm^{-3}).

Cumulated double bonds are when one atom has two double bonds, e.g. like =C=. Cumulated double bonds and triple bonds both occur in the Raman spectrum in the 2000 – 2200 cm^{-1} range [Smith & Dent, 2005].

ε — A material's **dielectric constant**, also known as relative permittivity, is the ratio of the permittivity of the material to the permittivity of free space. It is an expression for how effective it is for a material to concentrate electric flux. At optical frequencies it is a complex number given by $\varepsilon(\nu) = \varepsilon' + i\varepsilon''$, where ν is the frequency.

μ — **Dipole moment** measures the separation of two opposite charges. Its magnitude equals to the charge multiplied by the distance between the charges.

A — The **electromagnetic field enhancement** is defined as $|\mathbf{E}|/|\mathbf{E}_0|$, where $\mathbf{E}_0$ is the incident electrical field. According to the Mie theory, in vacuum, for a spherical nanoparticle, the electromagnetic field enhancement reaches maximum when $\varepsilon' = -2\varepsilon_0$. Metals like Ag and Au can significantly enhance the electromagnetic field due to their negative ε' and low damping, and are thus chosen extensively for SERS applications.

A **Notch filter** filters out light of same wavelength as the incoming light to avoid Rayleigh line from dominating the spectrum.

α — **Polarizability** is the ability to form instantaneous dipoles. It determines the dynamical response of a bound system to external fields.

SERS — **Surface-enhanced Raman spectroscopy** uses metalized, nanostructured surfaces to significantly enhance the Raman scattering signal of the analytes.

2.3 Raman spectroscopy

2.3a Light and its interaction with matter

Light is described either as waves of electromagnetic radiation or quantum mechanically as photons. The energy *E* of a photon is proportional to its frequency ν:

$$E = h * \nu, \qquad \text{(Equation I)}$$

where *h* is the Planck constant. $h = 6.626 * 10^{-34}$ Js.

When light encounters a sample, it is either; absorbed, transmitted or scattered. Absorption spectroscopy like infrared (IR) spectroscopy probes directly the energy gaps between the molecule's ground and excited vibrational states. Raman spectroscopy on the other hand deals with the in-elastically scattered light and probes indirectly the vibrational modes of the molecule. Most of the light is scattered elastically and has the same frequency as the incoming light. This process is called Rayleigh scattering. In Stokes Raman scattering however, the energy of the scattered light is lower than that of the incoming light, whereas in Anti-Stokes Raman scattering it is higher. Since Anti-Stokes Raman scattering requires the molecule to be in an already excited vibrational energy state, it is less frequent than the Stokes Raman scattering, which is normally presented in a Raman spectrum. Raman scattering constitutes about 10^{-8} of the scattered light, leading to molecular cross-sections in the order of 10^{-31}-10^{-26} cm^2 as compared to fluorescence spectroscopy with cross-sections of about 10^{-16} cm^2 per molecule [Smith & Dent, 2005; Kneipp, 2007]. The Jablonski diagram in Fig 2.3 illustrates the changes in molecular energies for IR absorption; Rayleigh scattering; Stokes and Anti-Stokes Raman scattering.

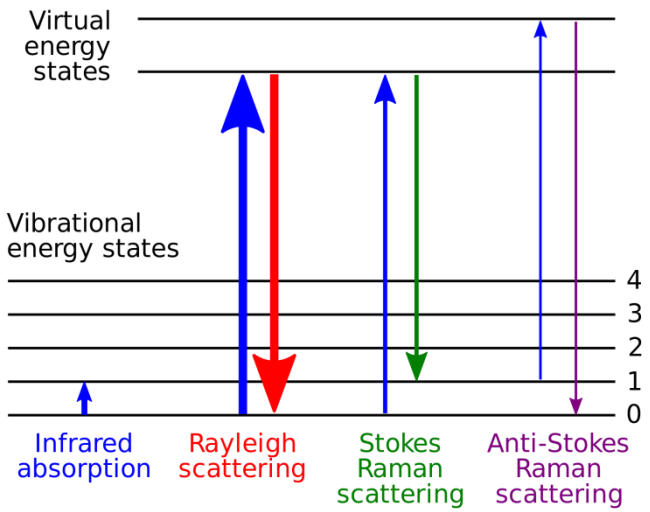

Figure 2.3: Jablonski diagram showing the energy states involved in infrared absorption, Rayleigh- and Raman scattering. In IR spectroscopy energy is absorbed, matching the induced vibration. In scattering processes the vibration is shifted to a "virtual" energy state after which the photons are re-radiated, either at the same frequency (Rayleigh) or at lower (Stokes) or higher (Anti-Stokes) energy levels compared to the incoming field [Figure copied from Wikipedia].

2.3b Polarizability and the Raman effect

The reason why some of the scattered light changes energy is that light can interact with a molecule by changing its vibrational state, whereby energy is consumed (Stokes) or taken up (Anti-Stokes Raman scattering). In a Raman scattering process, the incident light displaces the electron cloud surrounding the molecule and induces a dipole moment. Polarizability α determines the extent to which the electron cloud surrounding a molecule can be distorted by the incoming field. It is defined as the ratio between the induced dipole moment $\boldsymbol{p}$ and the electric field $\boldsymbol{E}$ producing it:

$$\boldsymbol{p} = \alpha * \boldsymbol{E} \qquad \text{(Equation II)}$$

α is often expressed in cgs units as polarizability volume in cm^3 or $Å^3 = 10^{-24}$ cm^3. Atoms increase in polarizability from right to left in the periodic table due to less filled outer electron shells. In order for Raman scattering to occur, the incoming light must change the polarizability of the molecule. Highly polar molecules like water cannot have their polarizability changed substantially, which is why they are not very Raman active. In a Raman scattering process, the incoming light interacts with the molecule through its polarizability, and introduces a dipole moment which is modulated by the molecular vibration. Consequently, the induced dipole oscillates not only at the frequency of the excitation light, but also at two side bands, emitting Stokes and anti-Stokes lights. The Raman selection rule is that α must change with molecular vibration, and the change is described by the polarizability derivative:

$$\delta\alpha/\delta\mathbf{Q} \neq 0, \qquad \text{(Equation III)}$$

where **Q** is the vibrational coordinate. When more atoms are present in the molecule, more vibrational modes are allowed. With N atoms in a molecule the possible number of vibrations is $3N$-6, except from linear molecules (like diatomic) where it is $3N$-5. Therefore a water molecule consisting of 3 atoms has 3 vibrational modes. Additionally, if the vibrational mode has a center of symmetry, it cannot be both IR- and Raman active. Therefore IR spectroscopy and Raman spectroscopy are considered complementary spectroscopic techniques. [Keresztury, 2002; Kneipp, 2013; Smith & Dent, 2005; Øgendal, 2011].

2.3c IR absorption

Unlike Raman-active vibrations, IR-active vibrations require that an electric dipole has an inherent asymmetric charge distribution. The dipole moment μ is defined as:

$$\mu = \mathrm{d} * \mathrm{q}, \qquad \text{(Equation IV)}$$

where d is the distance between the positive and negative charge q. The IR selection rule is:

$$\delta\mu/\delta\mathbf{Q} \neq 0 \qquad \text{(Equation V)}$$

So, for a vibration to be IR-active , the dipole moment must change during the vibration.

2.3d The Raman spectrum

A vibrational spectrum gives the signal intensity versus wave number $\tilde{\nu}$, which is usually expressed in the unit of cm^{-1}. The intensity of the Raman signal is the product of the number of molecules (N) in the probed volume, the laser intensity (I_L) and the Raman cross section (σ^R):

$$P_{RS} = N * I_L * \sigma^R \qquad \text{(Equation VI)}$$

A Raman spectrum gives Raman counts in time per unit of laser light, i.e. counts per second per mW as a function of the frequency shift away from the Rayleigh line, in wave numbers (cm^{-1}). Anti-Stokes Raman scattering would result in positive frequency shifts; but because the Anti-Stokes part of the spectrum is rarely used, usually only the Stokes Raman shifts are displayed in a Raman spectrum. For example, fig 2.4 shows a Raman spectrum of ambient air. The spectrum reveals the stretching modes of O_2 and N_2, respectively near 1600 cm^{-1} and at 2335 cm^{-1}. The stretching mode of N_2 is located at a much higher frequency than that of O_2. This is because N_2 has a lighter molar weight (28 g/mol) and a stronger bond (945 kJ/mol) in comparison to O_2 (32 g/mol and 497 kJ/mol).

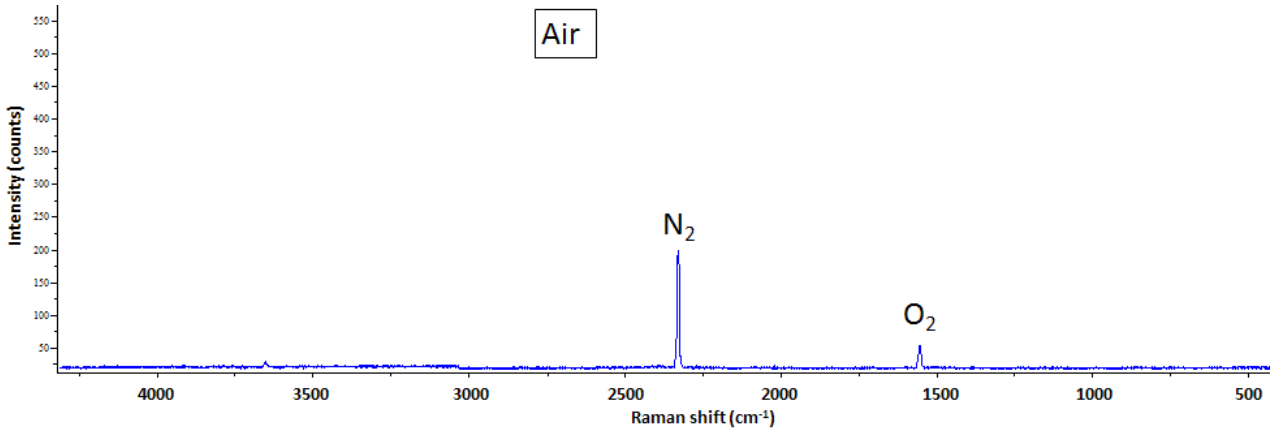

Figure 2.4: Raman spectrum of ambient air. The wave band of O_2 is found just below 1600 cm^{-1}, whereas the lighter and stronger bonded N_2 is located at a much higher frequency.

2.3e Surface-enhanced Raman spectroscopy

A major disadvantage of Raman spectroscopy is its low sensitivity due to the extremely small intrinsic Raman cross sections, which are 12-14 orders of magnitude smaller than those of fluorescence. Surface-enhanced Raman spectroscopy (SERS), using nano-sized metal particles is thus developed to enhance the Raman signal via surface plasmons (SPs). SPs are collective oscillations and redistributions of charges near the surfaces of metallic nanostructures, as is illustrated in fig 2.5. SPs generate electromagnetic hot spots which contain highly localized and enhanced electrical fields. In a hot spot, both the incident field and the Raman scattering field from molecules are amplified. The intensity of the SERS signal (P_{SERS}) is expressed by the following formula:

$$P_{SERS} = N * I_L * |A(\nu_L)^2||A(\nu_S)^2| * \sigma^R_{ads} \qquad \text{(Equation VII)}$$

N is the number of probed molecules. I_L is the intensity of the probing laser. $A(\nu_L)$ and $A(\nu_S)$ are the electromagnetic field enhancement factors for the laser and the scattered field. Since $\nu_L \approx \nu_S$, it can be deduced that the intensity of the SERS signal is proportional to the electrical field enhancement to the power of four. σ^R_{ads} is the effective Raman cross-section for the adsorbed molecule, and is known as the chemical SERS enhancement factor, which is typically 10 – 100 [Kneipp, 2007]. It is caused by electronic interactions between the molecule and metal. The chemical enhancement is regarded as a "first-layer effect". To trigger it, the distance between the molecule and the metal must be <10 nm.

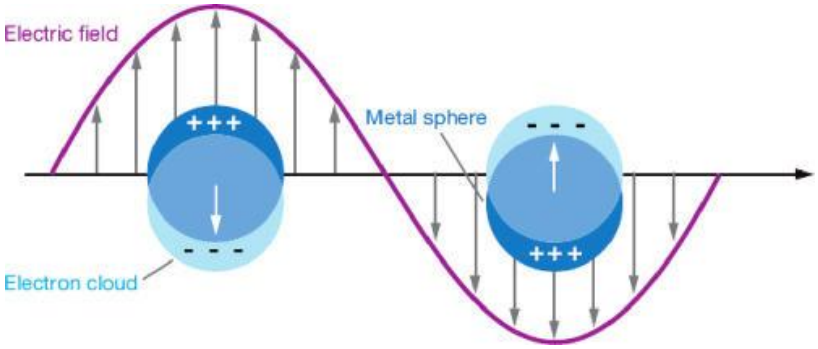

Figure 2.5: Illustration of surface plasmons (SPs). The negatively charged electric field passes metal nanospheres with unattached conduction electrons on the surface, oscillating opposite of the incoming electronic wave. [https://gregemmerich.wordpress.com]

Silver (Ag) and gold (Au) can create the most powerful surface plasmons and thus hot spots with the strongest electrical field enhancements, due to their negative dielectric constants and low loss. Electromagnetic SERS enhancement factors of higher than 10^8 are achievable. Therefore, surface-enhanced Raman spectroscopy could be ideally denoted "plasmon-supported Raman scattering" [Kneipp, 2007 and 2013]. The SERS substrate used in this project consists of Si nanopillars topped by Au caps with a total height of ~400 nm. When the nanopillars lean against each other, nanogaps are formed between the Au caps for hot spot formation and enhancement of the Raman signal [Schmidt *et al*., 2012].

2.3f Raman instrumentation

To perform Raman or SERS experiments, a monochromatic light source is necessary, which is often a 532 nm (green) or a 785 nm (near-infrared) laser. A typical configuration of a Raman instrument is shown in Fig 2.6.

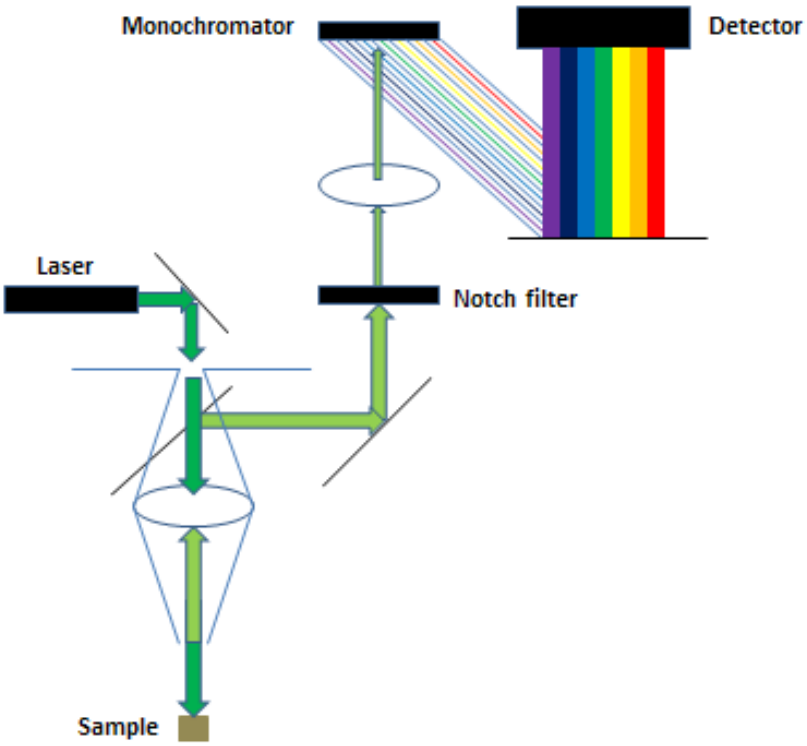

Figure 2.6: Configuration of a Raman instrument.

The excitation light passes through an aperture and is then focused on the sample by a lens. Afterwards, the scattered light is collected through the same lens, after which it, via mirrors, goes through a notch filter so that its Rayleigh component is eliminated. Subsequently, the light is focused onto a monochromator, where it is split up into its constituent wavelengths. Finally the light is collected by a CCD detector. For instruments with lasers of wavelengths above 1050 nm, another detector, such as InGaAs, needs to be used. In addition, when a microscope is used, it is necessary to limit the power density of the laser, because illuminating a very small surface area of a sample with a high laser power increases the risk of thermal degradation (burning). [Smith & Dent, 2005].

List of expressions – SERS substrate fabrication

e-beam evaporation	An electron beam melts the target metal, causing it to evaporate, to deposit a thin film of the metal onto the substrate.
RIE	Reactive Ion Etching; a (dry) plasma etch, forming trenches into silicon, used for micro- and nanotechnology. Longer etching time results in deeper trenches in the silicon [Jansen *et al*., 1995].
XPS	X-ray photoelectron spectroscopy. Machine for elemental analysis of a surface, using X-rays to free loosely bound electrons of a sample. The energy necessary to free the electrons is specific to every element.

2.4 SERS substrate fabrication

The SERS substrate used to enhance the Raman signal consists of silicon (Si) nanopillars ("nanograss") with Au caps and bases. When the pillars lean against each other, electromagnetic SERS hot spots are formed in the gap junctions between the Au caps. For illustrations, please refer to fig 2.7 and [Schmidt et al., 2012].

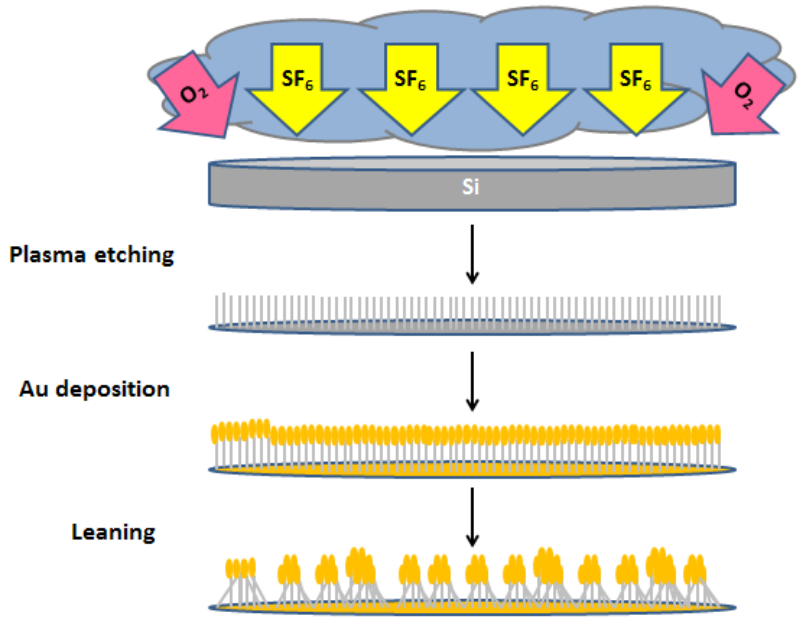

Figure 2.7: Preparation of the SERS substrate.

2.4a Nanopillar substrate

The nanopillars are made by reactive ion (plasma) etching (RIE) on a Si wafer. Depending on the etching time, Si nanopillars with different heights are formed. The employed RIE in this project consists of alternating SF_6 and O_2 plasma bombardment onto the Si wafer, respectively etching away Si and protecting the emerging nanopillars during the process. The RIE was done at -10°C. For the optimised substrate, three minutes of RIE was applied at a pressure of 36 mTorr, followed by 1 min O_2 clean. The O_2 clean step was included to get rid of SiF_x compounds which could be present on the substrate after etching, and it was important not to make the step too long, because then the metal would stick all the way down to the sides of the nanopillars, making them inflexible and difficult to lean [Kaiyu Wu]. Additionally, the SF_6 plasma can give rise to sulphurous substances, which can compete against cyanide for space on the Au surface [Lewis & Shaw, 1986].

2.4b Metal deposition

Using e-beam evaporation under vacuum, 225 nm thick Au was deposited onto the nanopillars at a rate of 9 Å/s. Immediately after metal deposition the wafer was hydrophilic, making the substrate easy to wet by a droplet added for leaning. If too long time passed, it would take longer time to dry because the droplet would not spread and become thin.

3.1 Project overview

The project consisted of 3 steps, of which the first was to establish a chemical method to detect cyanide on the substrate in relevant concentrations, preferably in gas. Step I was split up into two parts; one for HCN detection in the gas phase, and one for detection of potassium cyanide (KCN) in serial dilutions to reach sufficiently low CN concentrations and verify the limit of detection. Once this was done, the second step was to measure HCN(g) from emissions of *P. aeruginosa*; first from the established reference strain, the wild type PAO1. Secondly, it was relevant to study clinical *P. aeruginosa* strains, isolated from CF children for the first time in each patient (the wild type-like), and then compare to SERS measurements on later strains, isolated from the same patients after their infection became chronic and the *P. aeruginosa* had mutated in the *lasR* gene, which is essential to HCN production. Step III was a clinical trial, where children with CF would blow into a bag containing the SERS substrate, which was then measured, to see if HCN was detected; and data was correlated to sputum culturing. A schematic of the project flow is given in Fig 3.1.

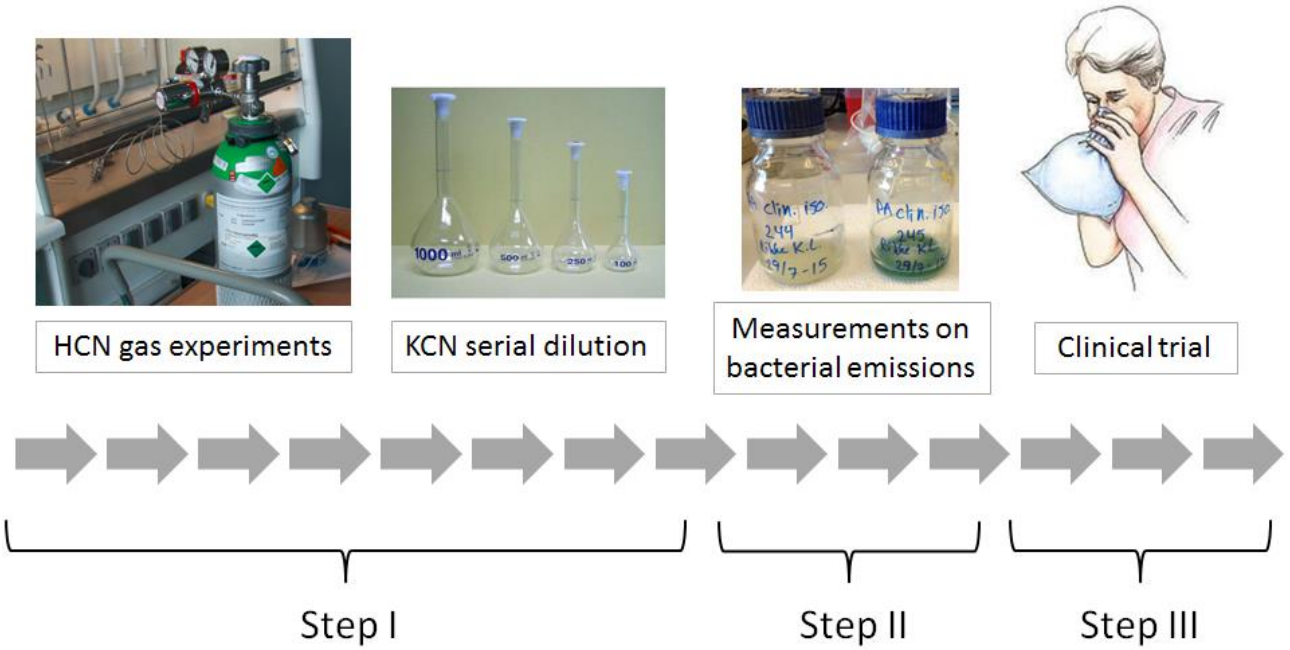

Figure 3.1: Overview of the project.

The outcome of each step was reported to a scientific journal, and the papers are found at the end of the thesis. Throughout the project, the SERS substrate was optimized for breath detection of HCN, which is presented in the next section.

3.2 Optimisation of the SERS substrate

3.2a Metal layer and cleaning procedure

When the NAPLAS project started, the recipe for the SERS substrate was 4 min RIE (no O_2 clean step), and the metal thickness was 200 nm (always Ag), deposited at a rate of 10 Å/s. Then a droplet of water was added for leaning, which could be distributed unevenly across the substrate. With experience it turned out that immersion was a better way to lean the pillars. Through the first 18 months, several experiments were made to compare Ag and Au substrates, knowing that HCN was used in Au mining and that the Ag substrates, although high enhancements, suffered from a poor shelf life due to oxidation, which could be a disadvantage for use including people. Cytotoxicity was not an issue, because it was never intended for physical contact. Different recipes were tried, varying etching time and cutting the wafers in halves for deposition of Ag and Au.

Because there was a background peak on the substrate close to the cyanide peak, addition of different O_2 and Ar plasma cleaning steps after the RIE were tried. Annealing the substrates at 400°C was also applied to try to minimise the background peak. Later, annealing was tried at 800°C in the cleanroom after RIE, and nothing helped. The only thing that improved the background a bit was a 1 min O_2 cleaning step immediately after the RIE. Out of the cleanroom the substrates were cleaned in ethanol, which also helped a bit. The cleaning procedure that helped most was immersion into analytical grade ethanol for 3 min followed by immersion into analytical grade water for 3 min (Fig 3.2), before the substrates were left to dry on a cleanroom tissue. This also induced pre-leaning which was good for gas measurements.

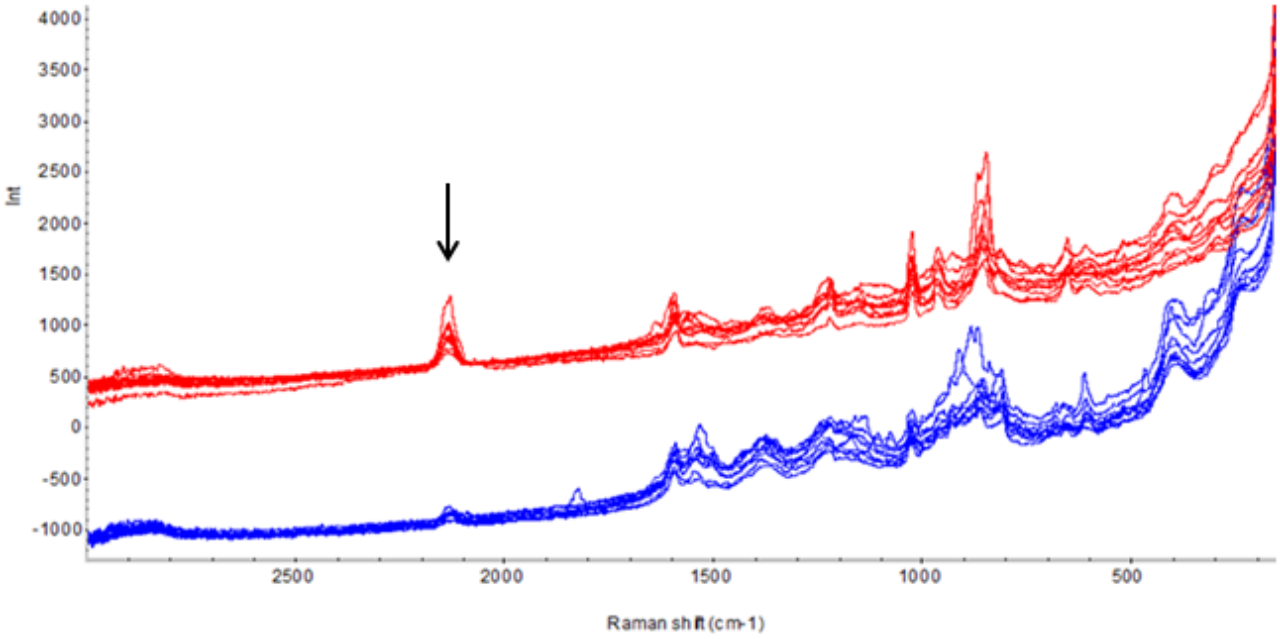

Figure 3.2: Comparison of no cleaning (Red) and 3 min ethanol followed by 3 min H_2O immersion (Blue) on the background peak at 2127 cm^{-1}, indicated by an arrow. After cleaning the peak is still present, but it is less intense.

For serial dilution experiments in aqueous KCN, the substrate was immersed into the particular dilution directly after the cleaning and rinsing procedure, and the exposed substrates were placed separated from each other on cleanroom tissues to dry and lean, to make sure they would not contaminate each other. For comparison of the cyanide peak intensity in KCN serial dilution experiments on the Ag and Au substrates, see Fig 3.3.

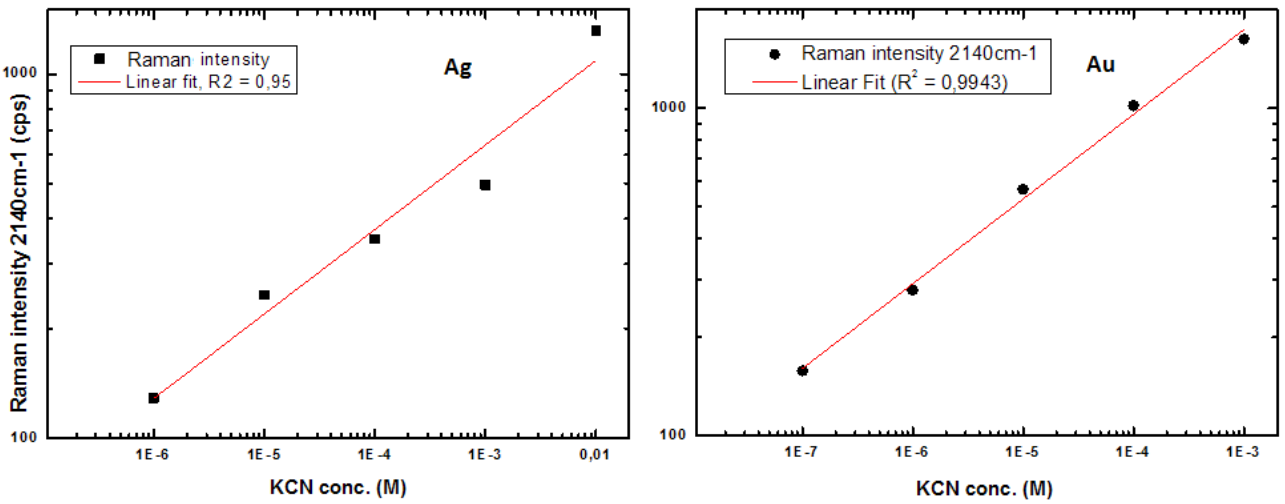

Figure 3.3: Serial dilution of KCN, comparing the 200 nm Ag and Au substrates. Note the logarithmic scales, on the X-axes respectively from 10^{-7}-10^{-3} and 10^{-8}-10^{-4}. It is seen that the Au substrate leads to C≡N peak intensities about twice as high as the Ag substrate.

Because the Au substrate seemed superior for the application, and due to the other advantages mentioned, it was decided to continue working only with the Au substrate. In order to see if higher enhancements could be obtained, various Au layer thicknesses were tried. The best seemed to be 225 nm:

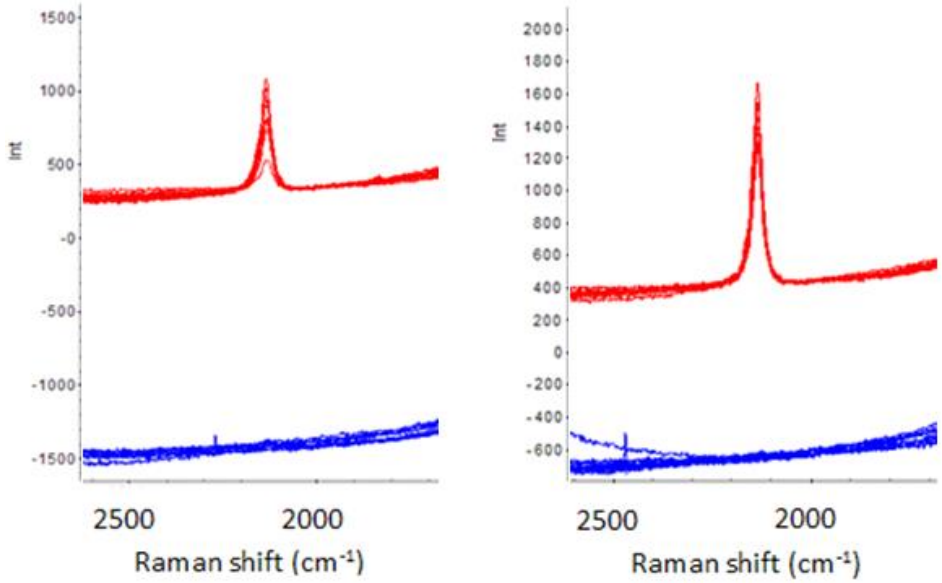

Figure 3.4: Comparison of SERS on 200 nm (left) and 225 nm (right) Au; 3 min. etch, 1 min. O_2 clean, before (Blue) and after exposure to 5 ppm HCN(g) (Red). The 225 nm substrate gives about double C≡N peak intensity of the 200 nm.

Double cyanide peak intensity of the 225 nm substrate, as compared to the 200 nm substrate was not always the case; but in general it performed better, which was why it was decided to be used instead. The substrates were made with 3 min etch, because this also gave better enhancement of the CN peak than the original 4 min etch. Reasons for this could be, that the longer pillars (due to longer etching time) collapsed easier, or that the shorter pillars (with shorter etching time) decreased the distance between Au caps and bases, which could be more optimal for SERS enhancement [Kaiyu Wu].

3.2b Attempts to identify the background peak

Carbon monoxide (CO) is also present in human breath and has a triple bond between C and O, and the stretching band could be very close to C≡N. Two times, bags of CO(g) were obtained from DTU Chemistry, and the gas was attempted for adsorption on the substrate, but without success. The background peak was unchanged, and the spectrum looked like the bare, cleaned substrate. According to Rae & Khan, in order to get CO to adsorb onto a AgPd SERS substrate it is necessary to first heat the substrate to at least 100°C and then cool it to 20°C before CO can be seen in the spectrum, so the background peak was probably not caused by CO [Rae & Khan, 2010].

The Si wafers are forming a thin glass layer (SiO_2) upon contact with atmospheric air [Jörg Hübner]. SiO_2 has a set of cumulated double bonds (O=Si=O) and could give a peak in the same region as C≡N. But the glass substrate being developed in the NAPLAS project does not have a peak there, so glass was probably not the cause. Due to the nitrile (-C≡N) bond it was checked whether the background peak could be caused by the nitrile gloves normally worn in the chemistry lab, so latex gloves (which were used in the cleanroom) were used instead; and this did not change the background peak.

The staff of DTU Danchip was helpful to make XPS measurements, identifying the surface elements at various stages of the procedure. First the bare Si wafer; then after etching (before and after N_2 blowing in the cleanroom); then after Au deposition (with and without the N_2 blowing). They were measured before and after cleaning and after immersion into 10^{-3}M KCN (in NaOH), respectively after exposure to 5 ppm HCN(g) in N_2. The XPS was checking for: Au, C, F, K, N, Na, O, S and Si. There was C in all samples, and additionally the following elements were found (Table 3.1).

Table 3.1: List of detected elements (besides C), using XPS during substrate fabrication and preparation.

	Si wafer	**Pillars – N_2**	**Pillars + N_2**	**Au – N_2**	**Au + N_2**
Uncleaned	Si, O	↑**O**,↓Si, F, S	↑**O**,↓Si, F, S	Au, O Int. 1.0×10^6	Au, O Int. 9.0×10^5
Cleaned		↑**O**,↓Si, ↓F, S	↑**O**,↓Si, ↓F, S	Au, O, ↑int. Int. 1.3×10^6	Au, O, ↑int. Int. 1.3×10^6
Cleaned + 10^{-3}M KCN				Au, **O**, Na, K, N Int. 1.1×10^6	Au, **O**, Na, K, N Int. 1.1×10^6
Cleaned + 5ppm HCN(g)				Au, O, (N) Int. 1.2×10^6	Au, O, (N) Int. 1.2×10^6

Carbon was present in all samples, and it is seen that after the Au deposition there was no trace of S or F on the substrate surface. In the cleanroom, substrates are often given a blow of N_2 before being further processed. Therefore, samples were also prepared for investigation with and without this N_2 exposure, and it is seen that this did not have any influence on the results. The cleaning procedure increased the intensity of what was seen on the surface, and N interacted better with the sample in liquid than in the gas phase, where pillars had been preleaned. There was more O in the immersed samples than in the ones exposed to gas, which was as expected because of O in the water and NaOH of KCN samples.

According to Jörg Hübner, low amounts of Si may diffuse into the Au, forming some sort of complex, but this was not detected from the XPS measurements. In summary, the background peak did not seem to be caused by CO, glass or nitrile. With Au on the pillar caps, no Si, S or F was detected on the surface, and N_2 blowing did not have any influence on the resulting elements on the substrate surface. The background peak remained unidentified, and more experiments were made to overcome the issue, e.g. deploying the fact that the C≡N peak, as a consequence of interaction with the Au surface, would shift to higher wavenumbers, away from the background peak.

3.2c Shelf life and ways to overcome the background issue

Shelf life was tested during the microbiological experiments. When there are sometimes problems with the machine depositing Au in the cleanroom, it is an advantage to know how old substrates can be used. An example is given in Fig 3.5, where new substrates with 225 nm Au deposited at 10 Å/s the day before measurements were compared to 17 days old substrates, where Au due to issues with the machine had been deposited to a thickness of about 205 nm at only 8-9 Å/s.

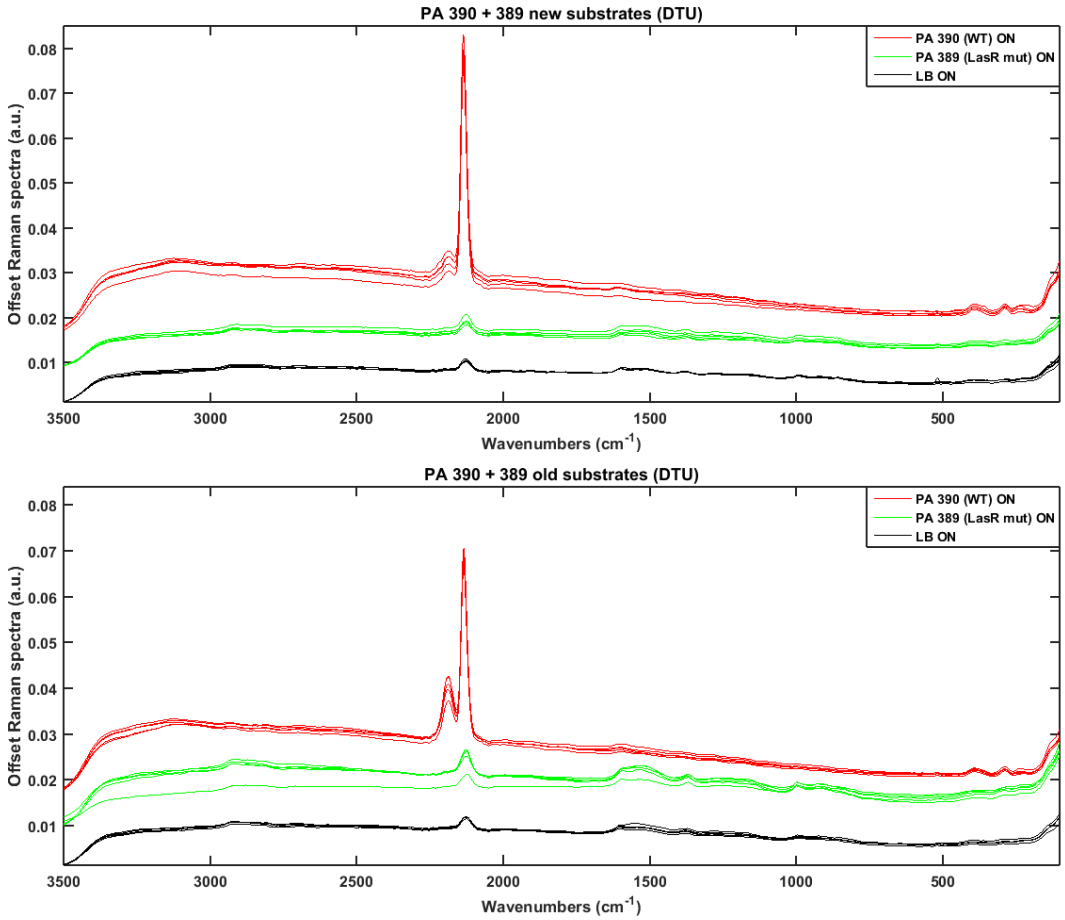

Figure 3.5: Comparison of Au SERS spectra of VOCs from *P. aeruginosa* 390 + 389 ON cultures on 1-day old substrates (new) and 17-days old substrates (old). Although slightly lower overall intensities in the old substrates, they seemed to be performing equally well. Old substrates had a lower deposition rate (8-9 Å/s) and about 205 nm Au, whereas the new ones had a deposition rate of 10 Å/s and 225 nm Au. It seems like the parameters of the "old" substrate promoted the cyanide peak's shift more than the parameters of the "new" substrate.

During metal deposition, it evaporates as atoms and can perhaps have time to diffuse together on the surface if the deposition is slow. If it is fast, the layer might become smoother [Silvan Schmid]. Altogether this should favour surface enhancement at lower deposition rates and perhaps ease the dissolution of Au, when the surface is rough. From the figure it is clear that, although slightly lower intensity, the parameters of the old substrate seemed to favour the C≡N peak shift (as explained in Paper I), which could be a huge advantage in moving the cyanide peak away from the background peak. The outcome was confirmed in seven other *P. aeruginosa* strains, deliberately comparing the deposition parameters of identical wafers broken into halves; and it was decided to change the deposition rate to 9 Å/S.

It was also clear that 17 days were not too long storage for the substrates to work in this application. Comparing two months old substrates to new ones showed that the 2 months old substrates were too old. Later, in the clinical trial, there was not sufficient HCN to make the peak shift, and the background peak again became an issue. Optimisation of the Au substrate took place throughout the entire project, but unfortunately, the background issue was never solved.

3.3 Chemical experiments (Step I)

3.3a HCN gas experiments

Even though 5 ppm HCN(g) is not very toxic, there was a long application process for getting permission to do the gas experiments in the lab. Rolf W. Berg from DTU Chemistry provided the flow cell seen in Fig 3.6, Kristoffer Almdal came up with the design, Søren M. B. Petersen realised the setup, and I applied for permission to use it. See Supplementary S1 and Paper I. Finally, permission was given for using the gas tank, mounted on a trolley, through a hole in the top table, in a safe lab with restricted access.

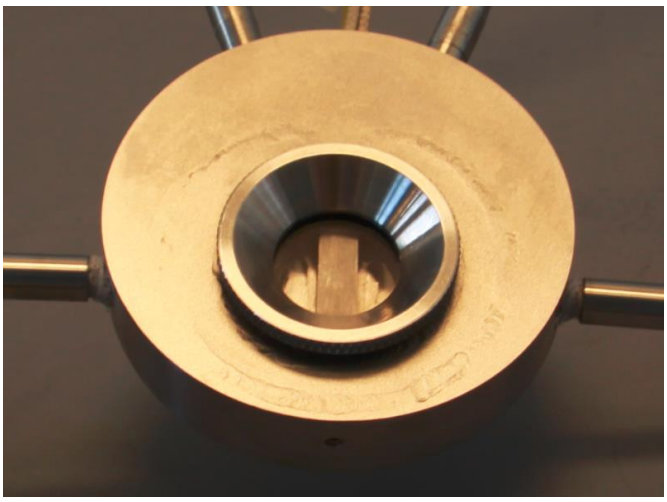

Figure 3.6: Flow cell for exposure of the SERS substrate to 5 ppm HCN gas in N_2. The O ring was turned, the loose glass slide dismantled, after which the chip was placed on the "bridge" in the middle, from where it could easily fall off when the gas was on.

The SERS substrate was optimised mostly during the first step of the project, and sometimes the Ag substrate was tested again to compare it to Au. In Fig 3.7, the optimised RIE recipe was used, and Ag, respectively Au was deposited at 5, 10 and 15 Å/s. It is seen that, although higher background triple bond peak, the Au substrate was much better suited for the application, and that 10 Å/s was the best deposition rate. For verification of cyanide on the substrate, two additional peaks were seen in the low frequency end, respectively assigned to Au-C stretching and Au-CN bending [Murray & Bodoff, 1986; Beltramo *et al*., 2003 Senapati *et al*., 2011]. At this time the gas exposure time had not been optimised, and 10 min flow through the cell was applied. Later it turned out that 30 s was sufficient, and through the rest of the project, 30 s gas samples were used as reference.

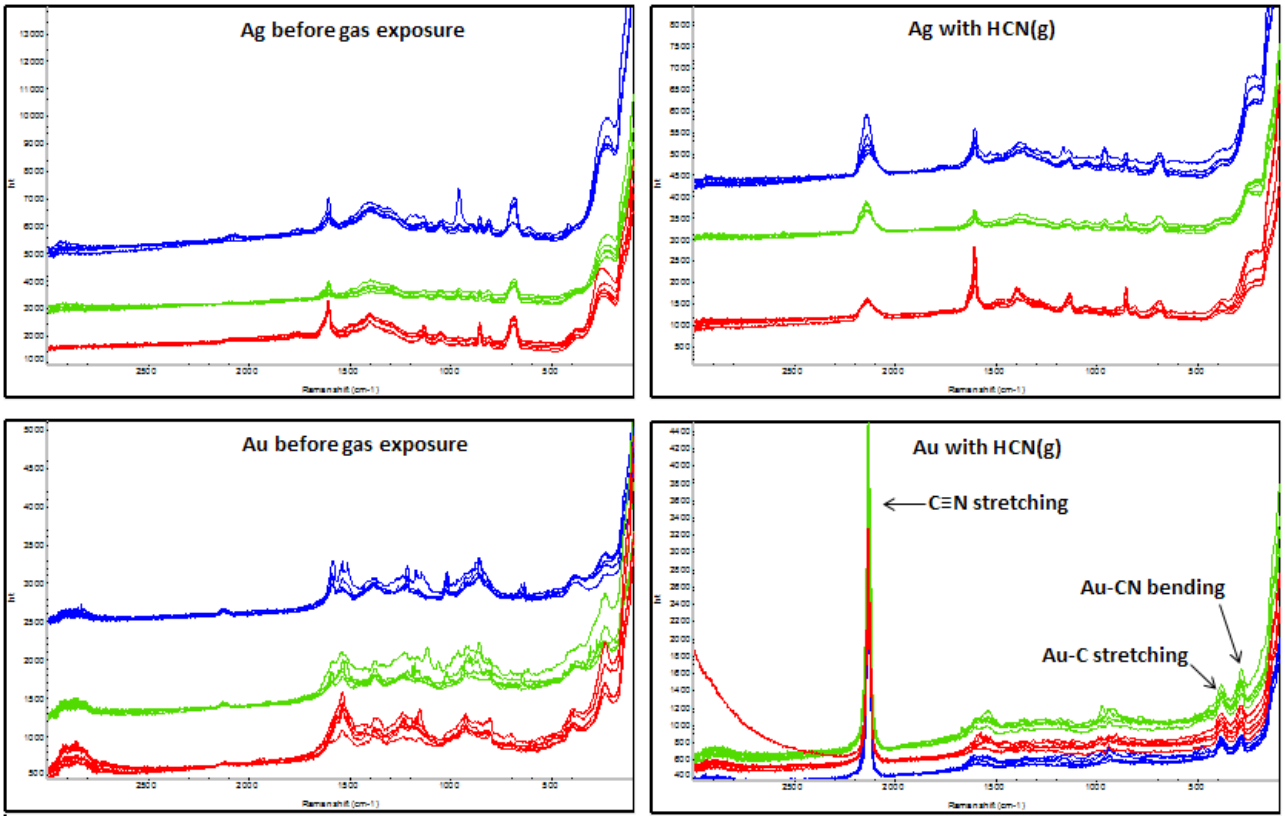

Figure 3.7: Comparison of Ag and Au substrates, 225 nm nanopillars, 3 min. etch, 1 min. O_2 clean. Deposition rate 5 Å/s (Blue), 10 Å/s (Green) and 15 Å/s (Red). Ethanol and water cleaned samples, exposed to 5 ppm HCN(g) for 10 min. Au substrates deposited at 10 Å/s are best suited for the application.

3.3b Serial dilution of KCN

HCN detection had been proven in the gas phase, although the concentration was about 1000 times too high compared to the breath of a child with a *P. aeruginosa* airway infection [Enderby *et al.*, 2009]. Therefore, serial dilutions of potassium cyanide (KCN) were made to see how low a limit of detection could be obtained. Since KCN is toxic, also through the skin or the eye, it was important to keep the lab clear to be able to focus on the task, and to have a structured plan to work by. Copies of the chemical risk assessment and a detailed work procedure are found in Supplementary S5. The serial dilution experiments were repeated between 8 and 10 times, first with droplets of the solution added to the substrate; and later with 3 min immersion into the alkaline KCN solution. A detection limit between 10^{-7} and 10^{-6} M was found, (Paper I). Because 10^{-4} M KCN resulted in SERS intensities of same order of magnitude as 5 ppm HCN(g); with 10^{-7} M detection, the desired ppb level was expected to be obtained, and it was time to move on to *in vitro* experiments, detecting HCN from emissions of *P. aeruginosa* cultures.

3.4 Microbiological experiments (Step II)

3.4a The setup

Optimisation of the procedure for microbiological experiments was done on the reference strain PAO1 in the Food Microbiology laboratory of University of Copenhagen (KU), Department of Food Science. The Raman measurements were also carried out at KU, in the Quality & Technology laboratory, Department of Food Science, where I went for my external stay in the period February-July 2015. Before going to KU, preliminary experiments with PAO1 were made at DTU Systems Biology, so that it would be possible to transfer the optimised setup to KU. There were, however, some differences between the procedures in the two labs:

1. At KU all broths, etc. should be prepared by oneself. From DTU, e.g. LB broth was taken from the shelf, and it had been prepared by the same lab technician who did this all the time. It also turned out that the recipes used were different in that at KU it contained 10 g/L NaCl, whereas at DTU it only contained 4 g/L salt, because it had been optimised for *P. aeruginosa* growth.

2. At KU they were not used to pathogens like *P. aeruginosa*, so in addition to the safety introduction I had to write a workplace risk assessment (APV) and 9 SOPs (see Supplementary S11 and S16), carefully planning how everything should be handled, before the lab work could begin. Another issue was the way ON cultures could be prepared, because I decided to use closed bottles shaking in the incubator to be sure not to spill bacteria and cause unnecessary worries to the group. At DTU, work with *P. aeruginosa* took place in every lab, and people were used to handle this as carefully as they should. Therefore, at DTU the ON cultures were incubated in flasks with two-holed rubber plugs shaking in an open incubator on the lab bench, which turned out to be much more convenient.

To get bacterial volatiles onto a substrate with the pillars pointing up, it was decided to use a pump and a vacuum chamber, as described in Paper II. Inside the vacuum chamber a reusable Teflon bag was placed, which was specially designed with one end open for mounting the SERS substrate. The bag was closed by a clamp and could be sterilized in an oven at 120°C for 30 min. Because ON cultures at KU were prepared in a closed flask (Fig 3.9), ON exposure had to be done before preparation of the time series, in order not to lose the vapours inside the flask when it was opened. Similarly, for each exposure of SERS substrates and Tenax tubes (see Discussion), one flask was prepared, because the bacterial emissions can only be used once. After exposing the SERS substrate to bacterial volatiles, OD was measured on each culture.

Parameter settings of the Raman were optimised for the 2135 cm^{-1} peak of a HCN(g) sample. Optimal spectra were obtained at 200 mW, 32 scans and a resolution of 8 cm^{-1}, and the C≡N peak position shifted to 2185 cm^{-1} during the measurements, as explained in Paper I. The 1064 nm laser gave less fluorescence at low frequencies compared to the 780 nm laser system at DTU Nanotech, which was an advantage because of some interesting peaks in this region (Paper I). Throughout the period of microbiological experiments, several attempts were made to establish a reference method for the quantification of HCN in bacterial gases, but they were never successful.

3.4b *P. aeruginosa* PAO1

The ON culture had to shake vigorously for growth to occur, because the microaerophilic bacteria need some O_2 to survive. 250 mL Bluecap bottles were used for the incubation of 10 mL ON culture, and the large volume above the culture turned out to be sufficient, although no extra air was let in. After confirmation of ON culture growth, a water bath and glassware were prepared. One mL of ON culture was added to 9 mL LB in a 100 mL Erlenmeyer flask which was plugged with a 2-holed rubber plug (Fig 3.8).

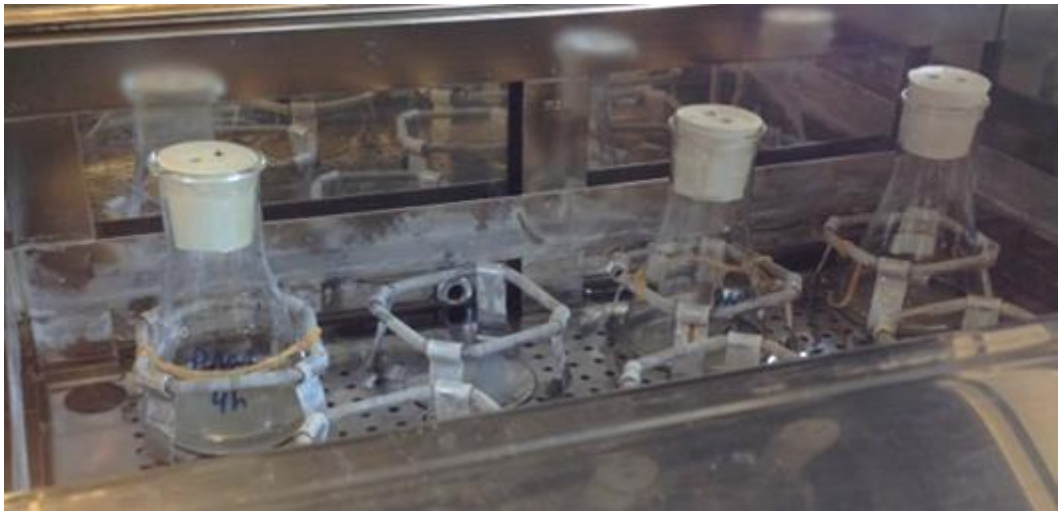

Figure 3.8: Fresh preparation of PAO1 time series. Time = 0, so the culture has not turned green yet.

From these flasks it was easy to expose the SERS substrates and compare the results across growth times and batches. This was not the case for the ON cultures. See Fig 3.9.

Figure 3.9: SERS exposure to ON culture emissions at KU.

As can be seen, a lid was turned so the tube from the vacuum chamber could come into the bottle. Problems were that bacterial emissions could be lost and there was no way to standardise the depth of the tube nozzle inside the bottle. Tape was put on the sides of the tube to minimise the opening, but it was not optimal, and the results were unreliable. Therefore, it was much better to use the Erlenmeyer flasks and

rubber plugs for valid substrate exposure, which was done in the PAO1 time series, except for ON measurements (Paper II).

As can be seen in Paper II, large amounts of HCN were produced from 4 hours' growth. Fig 3.10 shows measurements performed the day after exposure, illustrating how the C≡N peak shifts over time to the more stable $[Au(CN)_2]^-$ complex at 2187 cm^{-1}. It also shows a trend of an extra peak occurring at 238 cm^{-1}, which appeared after 6 and 8 hours' growth. The very large shift was only seen in quite "old" substrates, whose Au deposition had taken place about two weeks back.

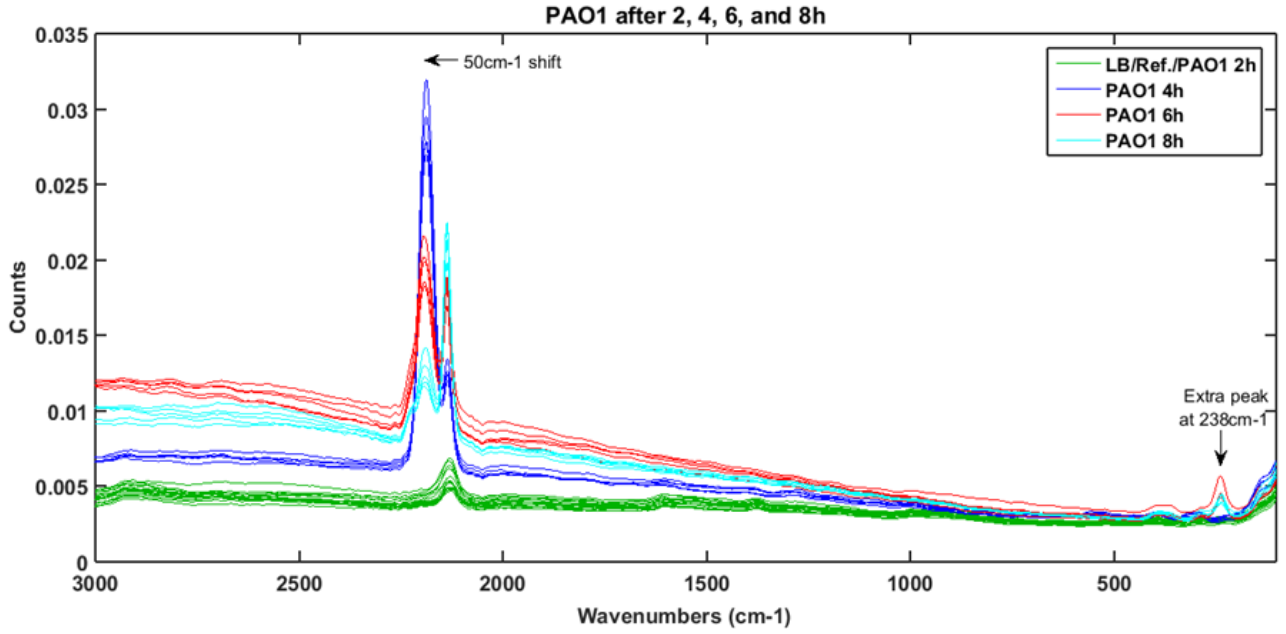

Figure 3.10: SERS spectra taken one day after exposure to PAO1 volatiles after 2; 4; 6; and 8 hours' growth. The 2h sample is identical to the LB and lab ref. samples. 4h shows the largest shift, followed by 6h and 8h samples, which had an additional peak at 238 cm^{-1}. For OD growth curves and "real time" HCN emission, the reader is referred to Paper II.

3.4c Clinical *P. aeruginosa* strains

Helle Krogh Johansen has made a collection of 500 *P. aeruginosa* strains isolated over time from 34 CF children followed at Rigshospitalet. From this collection, 13 strains of relevance to the project were selected. They were pairs of first isolate ("wild type (WT)-like") versus a later isolate from the same child after the infection had become chronic and adaptation with mutation in the *lasR* gene had taken place, among others coding for HCN production. From one child there was also an additional nonsense mutation. Isolates from one child failed to grow twice, so from the children collection 11 strains were examined. For comparison, the engineered *lasR* mutant of PAO1 (SD2) was used as well as the "classic" clinical CF strains DK02 from 1973 (WT-like) and the DK02 *lasR* mutant from 1979. An overview is summarised in Table 3.2.

Table 3.2: Summary of clinical and reference *P. aeruginosa* strains used in the investigation.

Reference strains				
		WT	***lasR* mutant**	
		PAO1	SD2	
Clinical strains				
CF no.	**Clone type**	**WT isolate no.**	***lasR* mutant isolate**	**no.**
114/30	02	IMG 140 (1973)	IMG 139 (1979)	
441	03	318.1	321	
544	06	13.1	10	
421	06	294	306	
499	06	390	389	
414	12	95	105	
382	32	244	245 nonsense	246 missense

Red marking means Produced HCN; Green means No HCN detected; Grey means No growth.

One mL freeze cultures prepared in glycerol were poured directly into 9 mL LB for clinical ON culturing. SERS substrate exposure of clinical strains were repeated in two labs; first in the Food Microbiology laboratory of KU; and then again in the Infection Microbiology Group (IMG) of DTU Systems Biology. All Raman measurements were made in the Quality & Technology laboratory of KU. Mutated isolates were not usually seen in the Food Microbiology laboratory, so ON measurements were made from the large Bluecap bottles. They were growing more slowly than PAO1, and since the experience from the PAO1 experiments was that HCN could still be detected in ON cultures (although in lower concentrations), only ON measurements were made. With time more experience was gained, and some of the results were valid, but all the experiments were repeated at DTU, preparing ON cultures directly in the Erlenmeyer flasks with rubber plugs. Results shown in Figs 3.11 through 3.13 are from DTU.

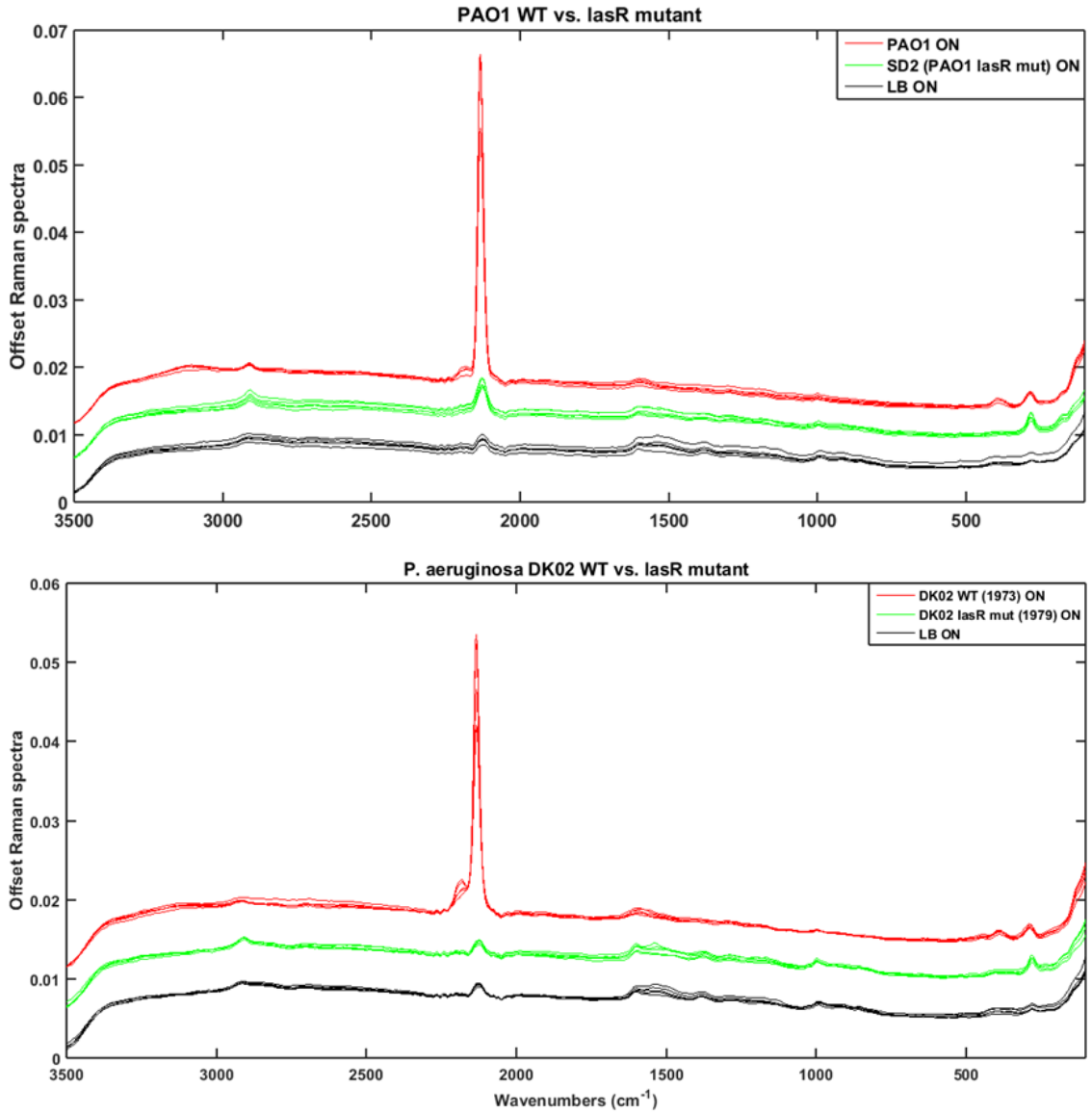

Figure 3.11: SERS on overnight cultures of reference strains. The wild type (-like) strains emitted high amounts of HCN, as can also be seen in the partly shifted C≡N stretching peak. No HCN was detected from the *lasR* mutated strains, where only the background peak was present. SERS on overnight LB growth medium is included for comparison.

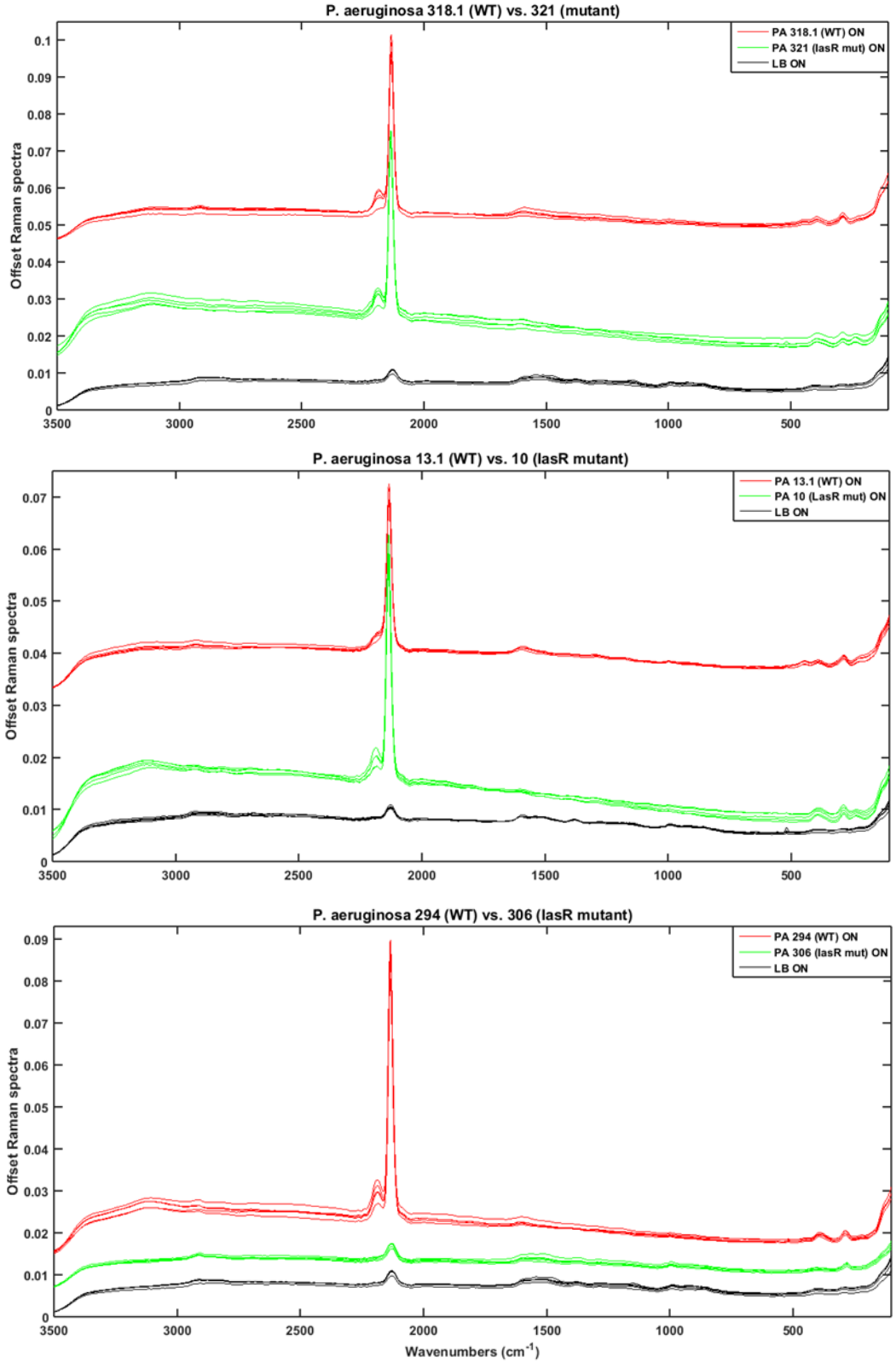

Figure 3.12: SERS on clinical overnight cultures from three CF children. All wild type-like strains emitted clearly detectable HCN, and the first two *lasR* mutants too. SERS on overnight LB is included for comparison.

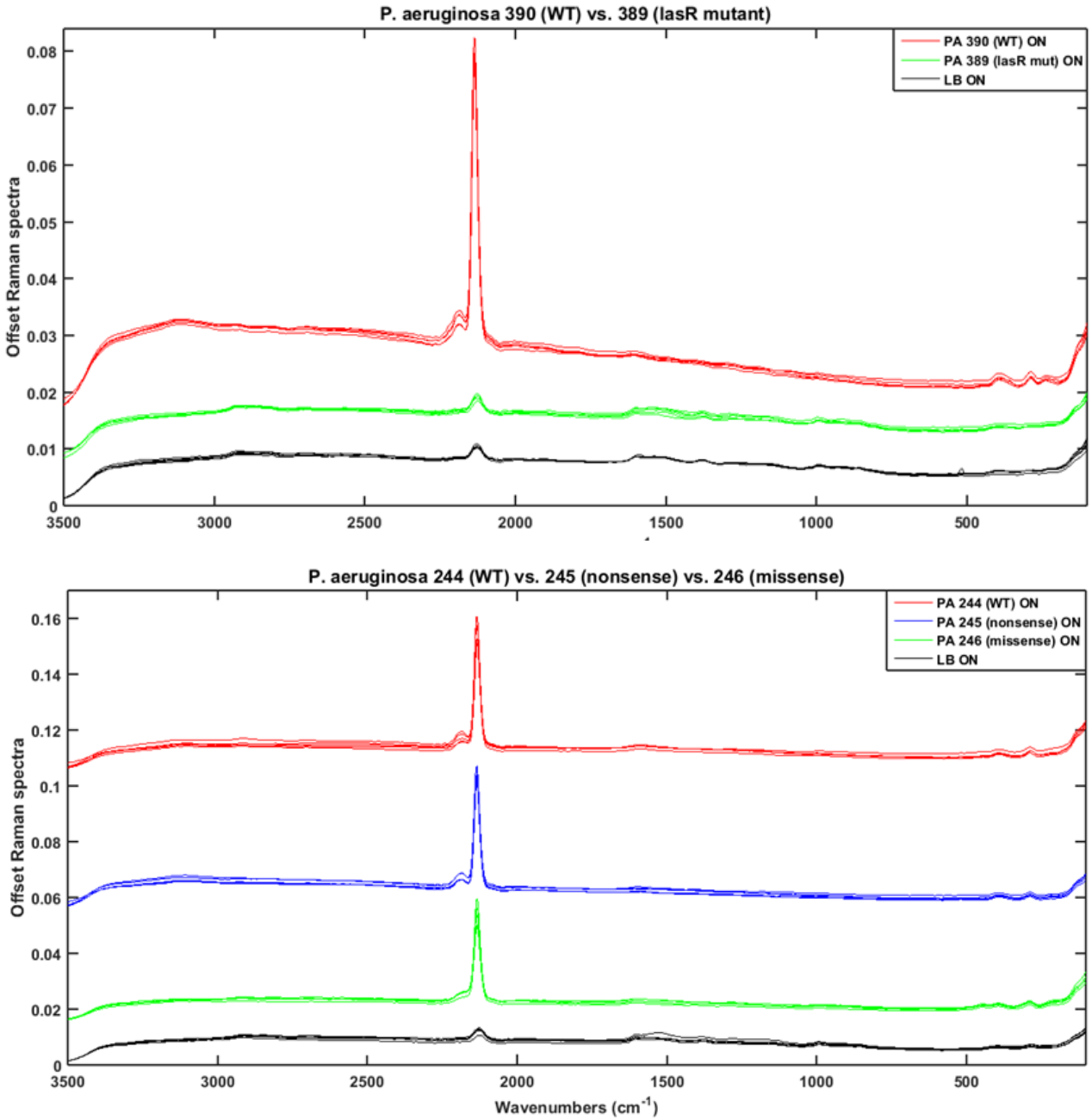

Figure 3.13: SERS on clinical overnight cultures from two children. The last is of a wild type-like; a nonsense *lasR* mutated; and a missense *lasR* mutated strain from one paediatric CF patient. Apart from isolate no. 389 (top Green), all strains emitted clearly detectable HCN. SERS on overnight LB is included for comparison.

All wild type-like strains emitted clearly detectable HCN, and also the *lasR* mutants, whose mutation was upstream (at the 5' terminal) of the gene. The *lasR* mutants, whose mutation was downstream (at the 3' terminal) of the gene, did not emit any detectable HCN (Paper II). Since, with the established SERS method, HCN could easily be detected from all WT-like cultures, the principle had been proven that the SERS substrate was possibly able to detect early *P. aeruginosa* infections from the vapours produced; so we applied the local ethics' committee and got permission to conduct a clinical trial, involving paediatric CF patients free from chronic *P. aeruginosa* infection.

3.5 Clinical trial (Step III)

3.5a SERS measurement on cigarette smoke

Tobacco smoke is known to contain HCN [Scherer, 2006]. To start the development of a device for collection of human breath, and to verify HCN detection from breath, experiments on cigarette smoke were made using Tedlar bags, known for their low background and FDA approved for human breath collection. The recycled bags used to collect bacterial volatiles were not relevant, because they were hand made from Canada and consisted of Teflon, which was impossible to weld, and the side welding was fragile. Tedlar bags are expensive, but strong, and they could be ordered in large quantities within short time. But they could only have one end open for mounting of the SERS substrate, if a corner was cut off, and the clamps from the Teflon bags were reused and disinfected with ethanol between collections.

Fig 3.14 shows SERS spectra of cigarette smoke blown directly into the bag containing the substrate; smoke through a PEP flutter with a SERS substrate mounted in the "chimney" of the PEP flutter's T-piece; and the smoker's breath into the bag after cigarette smoking. Even 3 days after exposure, the detection of HCN on the substrate was clear. Tobacco smoke also contains CO [Scherer, 2006], and due to the experiments referred to in 3.2b and the fact that the two extra CN peaks at the low end are seen in the spectra, it is expected to be HCN and not CO, which was detected on the substrate.

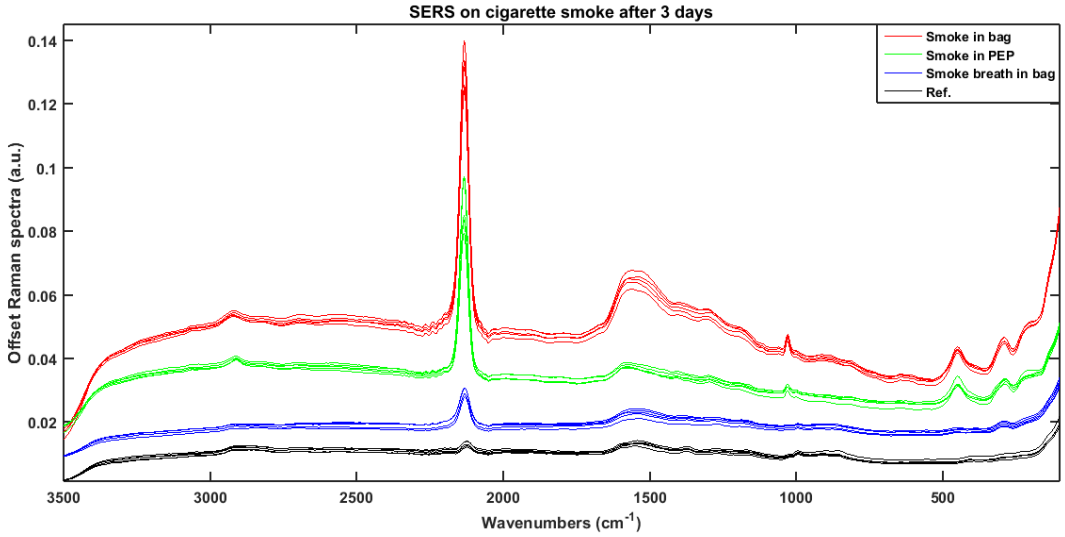

Figure 3.14: SERS on cigarette smoke measured 3 days after substrate exposure to smoke directly in the bag (Red), Smoke into a PEP flutter containing the SERS substrate (Green), the smoker's breath after finishing the cigarette (Blue), and a reference sample (Black). There is a clear C≡N stretching peak in the two first, and a smaller one in the smoker's breath. The 2 extra peaks < 500 cm^{-1} confirm CN detection.

Only 3 exhalations were used in the bag, whereas 6 exhalations were conducted through the PEP flutter, with similar HCN peak intensity, which led to the decision of using the bags, also so that extra exposure time could be applied. Additionally, it was easier to mount the substrate inside the bag than in the PEP flutter. And because successful experiments had already been made with the substrate mounted in a bag, it was decided that this was the best way to obtain SERS measurements on human breath.

3.5b Pilot study at Rigshospitalet

A protocol was agreed on with Kim Gjerum Nielsen (KGN), MD, from the Danish Paediatric Pulmonary Service and Helle Krogh Johansen (HKJ), MD, from Department of Clinical Microbiology at Rigshospitalet, including 50 CF patients and up to 20 controls aged 5-17 years. The age span of the patients should be relevant to the application, and they should be free from any *P. aeruginosa* infection, because the purpose was to catch a new colonisation, when it occurred. The lower age limit was set to be sure that the child was able to collaborate and blow correctly into the bag. Copies of the application to Ethics' Committee, along with information to patients and their parents; study protocol, informed consent form and questionnaire used, as well as a follow-up letter (all in Danish) are found in Supplementary S25. A very simple device was established for the collection of CF children's breath, connecting a Tedlar bag to a PEP flutter via a disposable tube. This way the child breathed through the PEP flutter, which he or she was familiar with for daily exercise, and into the bag containing the substrate for direct exposure. (Paper III).

Conducting a clinical study is a logistic task, because of the many stakeholders, patients, documents, machines and time plans to take into account. First of all, it took place in a normal hospital ward, with considerations and routines to comply with. CF children and their parents were waiting for their appointment with a doctor or nurse. Often they had been underway for a long time, because there are only two CF centres in Denmark (Rigshospitalet and Skejby). For young CF children, they knew that during the appointment, an endo-laryngeal suction would be made, and the child would be in for a small reward. Then came an extra person who would like to invite them to participate in a study on this "nano-chip" to try to find out, whether it could be used to detect *Pseudomonas* in the child's breath. The parents then had to read and sign the documents; and, depending on the infection status of the child, a consultation room was assigned, where the child was instructed how to blow into the bag. It had to take place before the child made the spirometry test (of lung function), because then the exhalation was forced as long as possible, and I was afraid to miss any bacterial HCN, that may have been produced in the airways.

Before this was possible, the SERS substrates were fabricated in the Danchip cleanroom of DTU, and every week Au had to be available in the machine for metal deposition, which should be up and running. Each morning, before patients entered the clinic, the substrates were cleaned and prepared in numbered Petri dishes; and devices and papers were prepared. The Petri dish was mounted in the Tedlar bag just before the child would blow into the device, and Petri dishes containing the SERS substrate were transported to the Quality & Technology laboratory, Department of Food Science at KU, for Raman measurements. The following week, microbiological results of the sputum culturing were available and imported to the case report form. Nanna Bild made this illustration to explain what the study was all about:

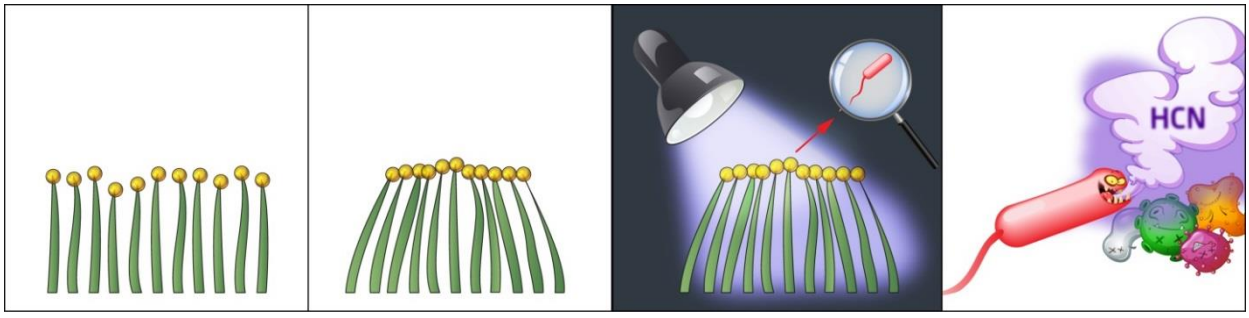

Figure 3.15: Illustration used to explain children and parents about the purpose of their participation.

The first month passed without any sign of HCN in the children's breath, and it was considered to pivot, using breath condensation instead and adding the condensate onto the substrate. Not even the child, whose *lasR* mutated *P. aeruginosa* isolate emitted HCN, seemed to have any HCN in her breath. If condensation was to be applied, it would be necessary to use strong base to make the cyanide stay in the condensate [Ma *et al*., 2010], which was not appealing; and the children seemed to like blowing up the bag, plus it would be a challenge to switch in the middle of the study. HKJ informed that the strain had been isolated 5 years back, so probably it had mutated even more since. This could explain why there was nothing in the patient's breath, because *P. aeruginosa* could have turned off the emission of HCN. It was therefore decided to persevere and continue according to the protocol.

Then an intense triple bond signal occurred, which can be seen in the Green spectra of Fig 3.16. Until then, all spectra had looked like the Red ones, which came from the same patient at his next visit.

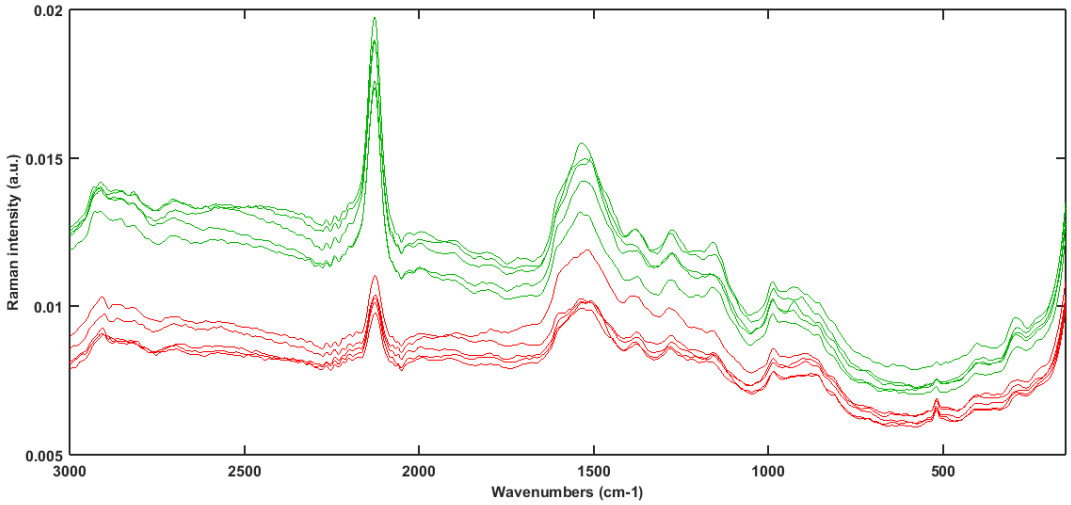

Figure 3.16: First indication of HCN detection in the breath of a CF child with a *P. aeruginosa* infection due to high intensity of the triple bond peak about 2130 cm^{-1} in the Green spectra.

The peak was at 2129 cm^{-1}, so it was not shifted. Whether or not HCN had been detected in the breath of the patient was uncertain, and it turned out that he had a non mucoid *P. aeruginosa* infection at both visits, and antibiotics had been administered in between. Because his precipitating antibodies against *P. aeruginosa* were 12 (and should have been < 2), he should not have been included in the first place.

Figure 3.17 is modified from Paper III. The SERS spectra came from a boy who cultured *P. aeruginosa* in his 3rd out of 4 visits. In the zoom it is seen that the triple bond peak was also slightly shifted away from the background peak, towards higher wave numbers.

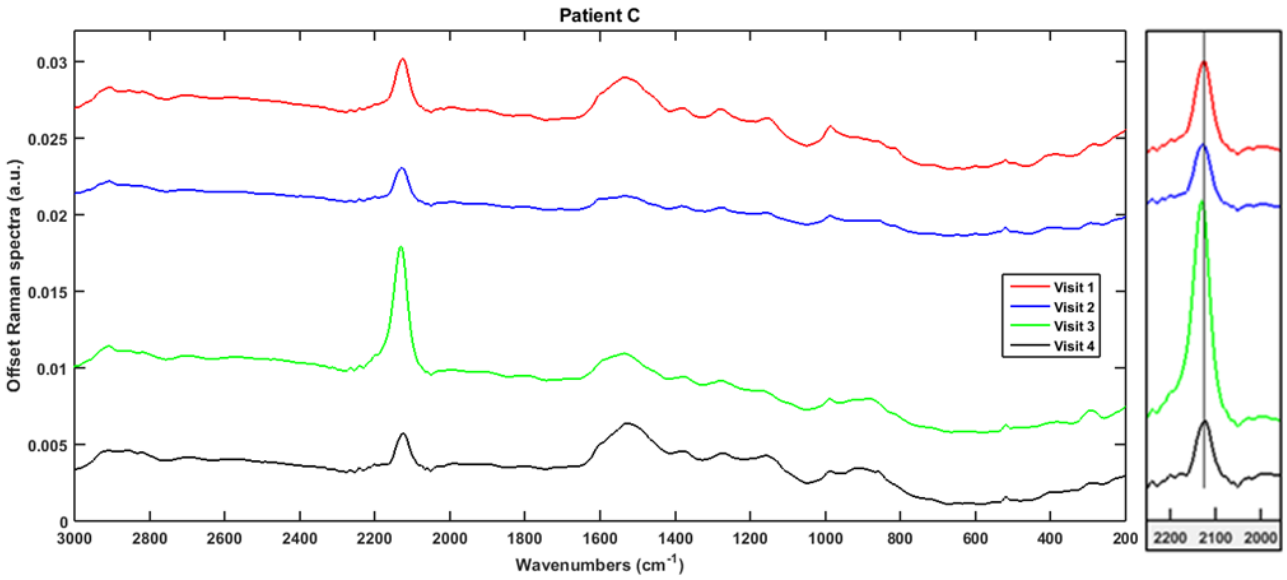

Figure 3.17: Mean SERS spectra of breath from patient C mentioned in paper III. The triple bond peak is more intense at the 3rd visit, where *P. aeruginosa* was cultured, and also slightly shifted above 2130 cm^{-1}.

Unfortunately, at the 3rd visit, the device had been left for 60 minutes before the substrate was evacuated. Because there was a probable dependence of exposure time, three bags were filled with breath of a healthy volunteer. See Fig 3.18. One was left for 15 min (Red), the second for 60 min (Blue), and the third bag was spiked to 20 ppb HCN(g) and left for 15 min. The scales of Figs 3.17 and 3.18 are identical, and it is seen that the Green spectrum of Fig 3.17 is more intense than the Blue of Fig 3.18 and as intense as the Green in Fig 3.18.

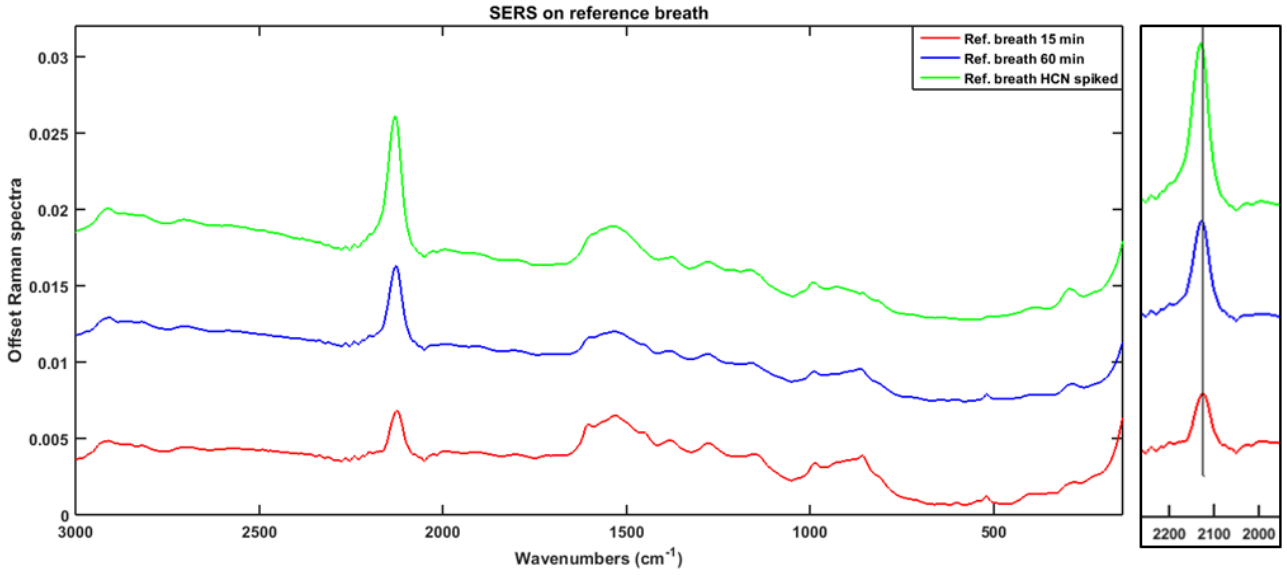

Figure 3.18: Mean SERS spectra of breath from a healthy volunteer. Red means 15 min exposure time, Blue is 60 min exposure time (as compared to the Green spectrum in Fig 3.17). The Green bag was spiked with HCN(g) to 20 ppb and left for 15 min. Raman intensity to the same scale as the previous figure.

Exposure time did have an influence on SERS intensity, but it did not seem to explain the entire enhancement of the triple bond peak of patient C's 3rd visit, because the Green spectrum of Fig 3.17 is more intense than the Blue of Fig 3.18, both representing mean spectra of 5 repetitions on the substrate.

A few of the very young control subjects were not blowing very well, probably because they were not used to force their exhalation, and this was reflected in their low intensity spectra. In about half of the spectra there was an additional peak at ~870 cm^{-1}, which was not assigned. It was speculated whether it could be caused by a small bottle of saline, which was often left open inside the laminar air flow (LAF) bench, where the substrates were prepared and left to dry, but this was not confirmed. It was also present in the spectra of visits 2 and 3 of another patient, in Fig 3.19 denoted patient D. The Red mean spectrum was from visit 1, the Blue from visit 2, and visit 3 was represented by two substrates; the Green of a Au substrate and the Black of a Ag SERS substrate, which was fabricated using the same RIE parameters. The Green and Black substrates were exposed at the same time, sitting beside each other in the same Petri dish, clearly demonstrating the superiority of the optimised Au substrate for this application. The Ag substrate had an intense O_2 peak about 1600 cm^{-1}, and a smaller CN peak, which on the Ag substrate was located at ~2144 cm^{-1}. The unidentified 870 cm^{-1} peak was also more pronounced in the Ag spectrum.

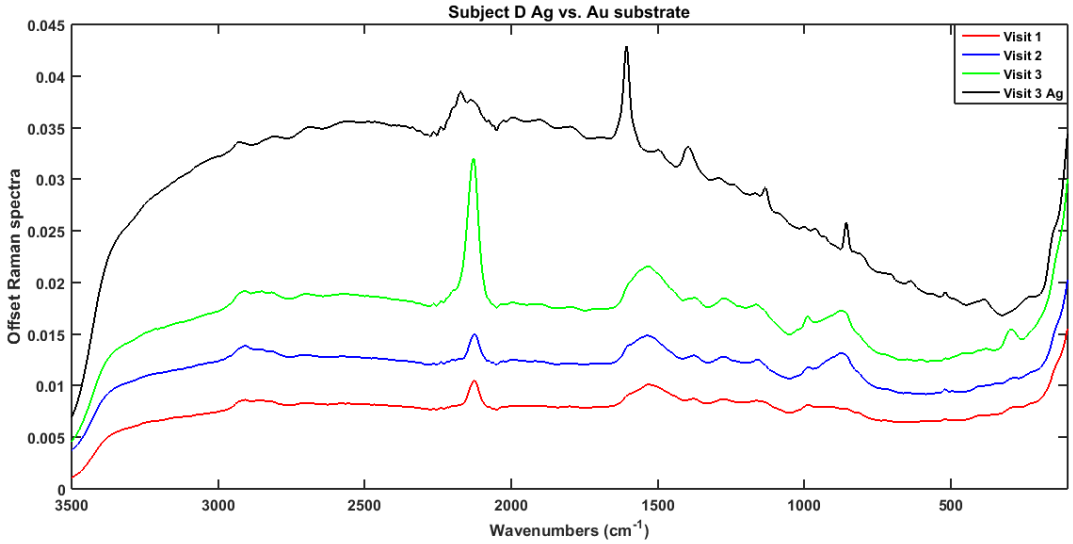

Figure 3.19: Mean SERS spectra on breath of patient D from 3 visits, the last both on Au and Ag substrates. Red from visit 1, Blue from visit 2, Green from visit 3 (Au), and Black from visit 3 (Ag).

The parents of subject D were divorced, and in the questionnaire of the third visit, the tick was set to "exposed to indoor passive smoking at home". At the first visit, it was at "not exposed to passive smoking at home". No *P. aeruginosa* was cultured from the patient, neither during the trial period, nor in the follow-up period of five months after the trial.

The results are preliminary, and more studies need to be made. Several other times the triple bond peak was intense, as explained in Paper III. One of the control subjects had an increased triple bond peak, and control subjects did not deliver sputum samples. Whether it was a *P. aeruginosa* lung infection, is therefore unknown. Although not a CF, the mentioned patient was too sick and should not have been included in the study.

3.6 Experimental summary

A nanopillar SERS substrate has been optimised for the detection of HCN in gas, in bacterial vapours, and in human breath. The substrate was invented previously, and the NAPLAS project founded [Schmidt *et al*., 2012]. The optimisation was done by changing the metal on top of the nanopillars from Ag to Au, lowering the etching time from 4 to 3 minutes and adding a one minute O_2 plasma clean step, lowering the metal deposition rate from 10 to 9 Å/s and increasing the thickness of the Au layer from 200 to 225 nm. Preparation for gas phase detection was done by preleaning the nanopillars, cleaning the substrates by 3 min immersion in ethanol followed by 3 min immersion in water and subsequent drying. The fragile substrates were kept and transported mounted inside small Petri dishes, tightly closed by small zipper bags.

Setups were developed for HCN(g) detection, for SERS detection of HCN from bacterial volatiles, and for collection and SERS substrate exposure to human breath. Five ppm HCN was successfully detected in gas phase, and KCN was detected down to 10^{-6} M. HCN detection was demonstrated from cultures of *P. aeruginosa* wild types, starting from the end of exponential / beginning of stationary growth phase. HCN was also detected from *lasR* mutated clinical *P. aeruginosa* strains isolated from the airways of children with cystic fibrosis (CF), when the mutation was located at the 5' terminal of the gene. Application for ethics' committee was submitted and permission given to conduct a clinical trial, including 50 CF patients aged 5-17 years and 19 age-matched control subjects. One CF patient had a new *P. aeruginosa* lung colonisation during the trial, and it was detected on the SERS substrate, whose HCN signal was increased compared to the patient's other visits.

Discussion

Hydrogen cyanide is a biomarker for the pathogenic bacterium *Pseudomonas aeruginosa*, which often causes chronic airway infections in CF patients. Using the silicon nanopillar substrate for surface-enhancement of the Raman signal, HCN was detected in the gas phase, although at too high concentration, because exact concentrations below 5 ppm could not be guaranteed. It was suggested to dilute the gas in the lab. But it was estimated that if the supplier could not promise exact concentrations below ppm, then with extra dilution it would be less certain how to conclude on the results both if HCN was and if it was not detected on the substrate. Median values of HCN concentrations measured in the breath of children with *P. aeruginosa* lung colonisations had been measured to 13.5 ppb [Enderby *et al*., 2009], so to be able to reduce the cyanide concentration, serial dilutions of potassium cyanide (KCN) were performed. For the chemical experiments, Raman measurements were performed on a 780 nm DXR instrument equipped with a x10 objective microscope. The microbiological and clinical Raman measurements were made on a BRUKER system equipped with a 1064 nm laser. Advantages hereof were that the spectra were less prone to fluorescence in the low end; that the BRUKER system was less booked and placed at KU, where the external stay took place and relatively close to Rigshospitalet; and because the project was prioritised, measurements could take place within short time after substrate exposure. However, due to repair, no objective was mounted on the instrument, and low Raman intensities were measured, and it is advisable in the future to measure using an objective to improve the efficiency of the SERS measurements.

Results on HCN production during growth of *P. aeruginosa* wild type were consistent with literature in that HCN emission was maximal at the end of exponential or beginning of stationary growth phase [Castric, 1975; Blier *et al*., 2012; Chen *et al*., 2016]. The clinical WT-like strains also emitted high amounts of HCN, which could still be detected in ON cultures. Since initial colonisation usually occurs with a non-mucoid strain [Smith *et al*., 2013], this was in agreement with reports of five times higher HCN emission in non-mucoid clinical strains isolated from CF patients than in non-mucoid laboratory strains [Carterson *et al*., 2004]. Since a functional *lasR* gene is essential to HCN production by *P. aeruginosa* [Pessi & Haas, 2000], it could be expected that *lasR* mutant strains would not emit HCN. This was the case in the *lasR* knocked-out strain SD2, and in the clinical 1979 reference strain from the DK02 lineage, which is a "classical" *lasR* mutant [Damkiær *et al*., 2013]. Therefore, it was surprising that some of the *lasR* mutated isolates from the children collection actually produced HCN. Genome sequencing had previously been made on the isolates from CF children [Marvig *et al*., 2015], and it turned out that the location of the *lasR* mutation played a role, in that mutations downstream of the *lasR* gene lead to cessation of HCN production, whereas upstream *lasR* mutation did not seem to have any influence on HCN production. This was in accordance with Kiratisin *et al*., who suggested that the LasR protein has two regions, of which the C-terminal region (downstream) promotes or strengthens multimerisation, which is its way of action. When this part was mutated, then probably therefore no HCN was synthesized [Kiratisin *et al*., 2002].

The microbiological experiments were followed up by a clinical pilot study, where two CF patients who had delivered *P. aeruginosa* strains to the *in vitro* experiments were also tested *in vivo*; but no HCN was detected from their breath. Using SESI-MS, only 25-34% of the volatile *in vitro* peaks of *P. aeruginosa* were shared with breathprints *in vivo*, which is why it is important not to evaluate *in vitro* volatile fingerprints

based on *in vivo* assumptions [Zhu *et al*., 2013b]. One new *P. aeruginosa* colonisation occurred during the pilot study, and the SERS C≡N peak was more intense than in the spectra from the patient's other visits, where only the background peak was present. Too long exposure time had been applied to the SERS substrate at the visit, where sputum culture was positive, and experiments were made on the breath of a healthy control subject, comparing normal (i.e. 15 min) exposure time to prolonged (60 min) exposure time. The C≡N peak was more intense after 60 than after 15 min, but not as intense as in the *P. aeruginosa*-positive SERS breath spectrum, and also not as shifted above 2130 cm^{-1} as this sample, whose C≡N peak was comparable to healthy breath spiked to ~20 ppb HCN. Additionally, there were more occasions of increased CN peak intensity, and for the test to be valid, the false positives must be reduced to a minimum. It is important to note, that it is a very sensitive substrate, and possible alternative sources of cyanide, triple bond or cumulated double bond compounds need to be investigated. The results are preliminary, and more pilot studies and clinical trials are recommended to come up with more concrete statements.

Working with a novel method, which has a background peak close to the analyte peak, it is relevant to have a valid reference method to be sure about the conclusions drawn. Measurements on HCN(g) and KCN in dilution had given information about the SERS spectrum of cyanide and how it behaved at different concentrations. But working in a microbiological setting, where other factors could play a role, it was important to be able to validate that the peak expected to occur from cyanide actually did, because the background peak was only a few wavenumbers away. At the Novo Nordisk Foundation, Centre for Biosustainability, DTU, a well-established GC-MS method was used to quantify bacterial volatiles, adhered to Tenax. The learning was that HCN cannot stick to Tenax, and that regular GC-MS methods cannot go below molecular masses of 30 g/mol. In attempts to find another suitable chemical or GC-MS reference method, more people were approached, but no reference method was established.

When sputum was cultured, ideally it should originate from an area where infecting microorganisms were growing. Since they thrive in clots, this might not always be the case, and so the culture would be negative. Once a sputum sample was obtained, it was transported to the Department of Clinical Microbiology who would culture the sample. Depending on internal mail routes and routines this could take up to 24 hours; often under sub-optimal conditions for the bacteria to survive, and in the meantime some of them might die. This could imply that the culture would be a false negative, and the bacteria could continue to develop in the patient's airways until next visit to the outpatient clinic, where they might be discovered.

It could be argued that the project has been moving too fast and that more knowledge should have been gained within each step before moving on to the next. But the aim of the PhD project was to investigate the applicability of the method to a new field, and when results were convincing and consistent, it was time to move on to the next level, although it was not completely understood, e.g. exactly what caused the background peak. Even though it would have been a huge advantage to solve the issue, it was outside the scope of the PhD project. Ideally, optimisation of the Au substrate should have been more advanced before or during the course of the NAPLAS project, whose main focus was on optimising the Ag substrate. Since then, the RIE recipe for the Au substrate has been optimised further, among other things by lowering the chamber pressure for increased density of the nanopillars [Kaiyu Wu].

Conclusion and outlook

The SERS substrate has been taken from basic research, through *in vitro* studies, and into a clinical setting. It can be concluded that the substrate has been improved and is able to detect HCN from human breath. With time, it may hold the potential to be developed into a point-of-care diagnostic tool, which, together with a portable Raman system, could be placed in the patient's home or at the general practitioner for daily breath testing of the *P. aeruginosa* biomarker HCN. For this to be realised, the issue with the background peak close to the CN peak needs to be addressed, and a standard way of exhaling should be established, possibly using condensed breath collected onto the substrate, which should then be alkaline. A disposable device (the R-tube) for breath condensation is available from MESM, Ltd.

It is recommended to conduct more clinical studies, applying different approaches, testing for longer periods, possibly also including PCD patients, and including control subjects who are less sick. As reference method, instead of sputum culturing, it is recommended to use PCR to upscale the DNA of the sample for more sensitive microbiological diagnostics. Until this is applied, the sputum samples should be stored in a better way and preferably transported to the Department of Clinical Microbiology right away, and at least same day as the sample is obtained, so there is a better chance of culturing possible microorganisms in the sample.

With new drugs on the market, targeting the ion channel defects of the delta F508 and the third most common *CFTR* mutation, G551D; there is hope for a cure to CF, if the annual price of $259,000 or almost 2 million DKK per patient could become more accessible.

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Paper I

”Towards quantitative SERS detection of hydrogen cyanide at ppb level for human breath analysis”

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Towards quantitative SERS detection of hydrogen cyanide at ppb level for human breath analysis

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ABSTRACT

Lung infections with *Pseudomonas aeruginosa* (PA) is the most common cause of morbidity and mortality in cystic fibrosis (CF) patients. Due to its ready adaptation to the dehydrated mucosa of CF airways, PA infections tend to become chronic, eventually killing the patient. Hydrogen cyanide (HCN) at ppb level has been reported to be a PA biomarker. For early PA detection in CF children not yet chronically lung infected a non-invasive Surface-Enhanced Raman Spectroscopy (SERS)-based breath nanosensor is being developed. The triple bond between C and N in cyanide, with its characteristic band at ~2133 cm^{-1}, is an excellent case for the SERS-based detection due to the infrequent occurrence of triple bonds in nature. For demonstration of direct HCN detection in the gas phase, a gold-coated silicon nanopillar substrate was exposed to 5 ppm HCN in N_2. Results showed that HCN adsorbed on the SERS substrate can be consistently detected under different experimental conditions and up to 9 days after exposure. For detection of lower cyanide concentrations serial dilution experiments using potassium cyanide (KCN) demonstrated cyanide quantification down to 1 μM in solution (corresponding to 18 ppb). Lower KCN concentrations of 10 and 100 nM (corresponding to 0.18 and 1.8 ppb) produced SERS intensities that were relatively similar to the reference signal. Since HCN concentration in the breath of PA colonized CF children is reported to be ~13.5 ppb, the detection of cyanide is within the required range.

1. Introduction

Cystic fibrosis (CF) patients have a salt imbalance of the secretory cells leading to excessive, salty sweating and swelling of the pancreatic duct, accompanied by an insufficient uptake of proteins and lipids. The most commonly known symptom is swelling of the bronchial wall due to vastly dehydrated and thickened mucus and bacterial infections, leading to coughing and loss of breath. Bacteria often infect the sinuses, and it has been shown that they adapt to the lower airways in the sinuses and drop into the lungs e.g. during sleep or viral infections [12,15]. The bacterium most commonly associated with morbidity and mortality in CF patients is *Pseudomonas aeruginosa* (PA) which is harmless to healthy individuals but well-adapted to the oxygen depleted environment in the upper airways [1]. The intermittent PA lung colonization often becomes chronic, which is why early detection and eradication is essential [19].

P. aeruginosa emits the poisonous gas hydrogen cyanide (HCN) to outmatch competitive microorganisms, and HCN has been suggested as a potential PA biomarker [4,9,16]. Selected ion flow tube mass spectrometry (SIFT-MS) and other MS based principles are among the most frequently applied techniques for human breath analysis, detecting HCN levels between 0 and 81 ppb [2,7,9–11,21,29]. Because HCN is also formed in the oral cavity of adults it has been suggested to only use nose exhaled breath for analysis in adult CF patients. A "cut-off" value of 10 ppb has been suggested as "elevated", indicative of a PA infection [10]. In the breath of CF children with a PA airway colonization HCN has been reported to lie between 8.1 and 16.5 ppb, with a median value of 13.5 ppb [9]. For a definition of PA colonization versus chronic infection please refer to [14].

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For mouse model systems secondary electron spray ionization mass spectrometry (SESI-MS) has been used for *in vivo* breath diagnosis of lung infection models [30,31]. The authors point out that *in vitro* studies cannot be expected to mimic *in vivo* results because only between 25% and 27% of the *in vitro* and *in vivo* PA peaks were shared. For analysis of sputum and broncho-alveolar lavage (BAL) samples various electrochemical probes have been used to measure cyanide content at micromolar level [3,11,22,27]. The techniques for breath analysis are either, expensive, inflexible, time consuming or they demand a high level of operator skills [5]. Therefore, there is a need for a fast, inexpensive and sensitive sensor for the detection of *P. aeruginosa* in the breath of children with cystic fibrosis.

Raman scattering spectroscopy is a widely used fingerprinting method for small molecules. However since it is not a very sensitive method, millimolar concentration seems to be the limit of detection [6]. Surface-Enhanced Raman Spectroscopy (SERS) using gold nanoparticles in a sol–gel has been used to detect ppb level cyanide in waste water [20], and in 2011 Senapati et al. reported to have detected cyanide in the ppt range by the use of Au SERS aggregation [26]. In the presence of noble metals, cyanide can be detected due to its high affinity towards metals. These approaches demand cyanide to be in solution for SERS detection and there is much sample preparation. We propose a fast and cheap technique based on the SERS substrate developed by Schmidt et al. [24] that can be used both in solution and for the direct detection of cyanide in gas phase as a precursor for PA breath detection. The substrate consists of gold coated silicon nanopillars which can be brought to lean against each other, forming so-called "hot spot" regions with considerate plasmonic effect for Raman signal enhancement to take place. It is well-known that Raman is a quantitative method [25] and in some cases it is also possible to perform quantitative SERS, although it has yet to be demonstrated to a wider extent [8].

Hydrogen cyanide is a potential PA biomarker. If a point-of-care device could detect HCN in the breath of young CF patients with a pulmonary PA colonization, the need for invasive techniques and repeated anesthesia for obtaining broncho-alveolar lavage to diagnose PA would be minimized. To make a simple model system for the presence of HCN in breath, a gas setup with an open flow cell connected to a tank of 5 ppm HCN(g) in N_2 was used. The SERS substrate was placed inside the flow cell and exposed to the gas for 30 s. To vary the amount of HCN molecules exposing the substrate, the pressure through the open system was changed among samples.

In the present paper SERS measurements on 5 ppm HCN gas and on serial dilutions of potassium cyanide (KCN) in the region from 10 nM to 1 mM are presented. A KCN concentration range of 100 nM to 1 μM is the region of relevance, corresponding to ppb gas levels. The aim of the project is to detect PA colonization in the patients' breath at an earlier stage than allowed by today's conventional methods.

2. Experimental

2.1. SERS substrate fabrication and measurement procedure

Nanopillars were etched into a Si wafer in an Advanced Silicon Etcher, applying alternate portions of SF_6 and O_2 plasma (dry etch), followed by an O_2 cleaning step. The resulting nanopillars are 400 nm in height, 50 nm in width, and with a density of approximately 18 pillars/μm^2. A 225 nm thin Au layer was then deposited onto the silicon nanopillars, producing freely standing Au-capped Si nanopillar structures (Fig. 1(a)). The wafer was diced into 6 × 6 mm^2 squares using a diamond cutter and the substrates were used within 1–2 days after Au deposition.

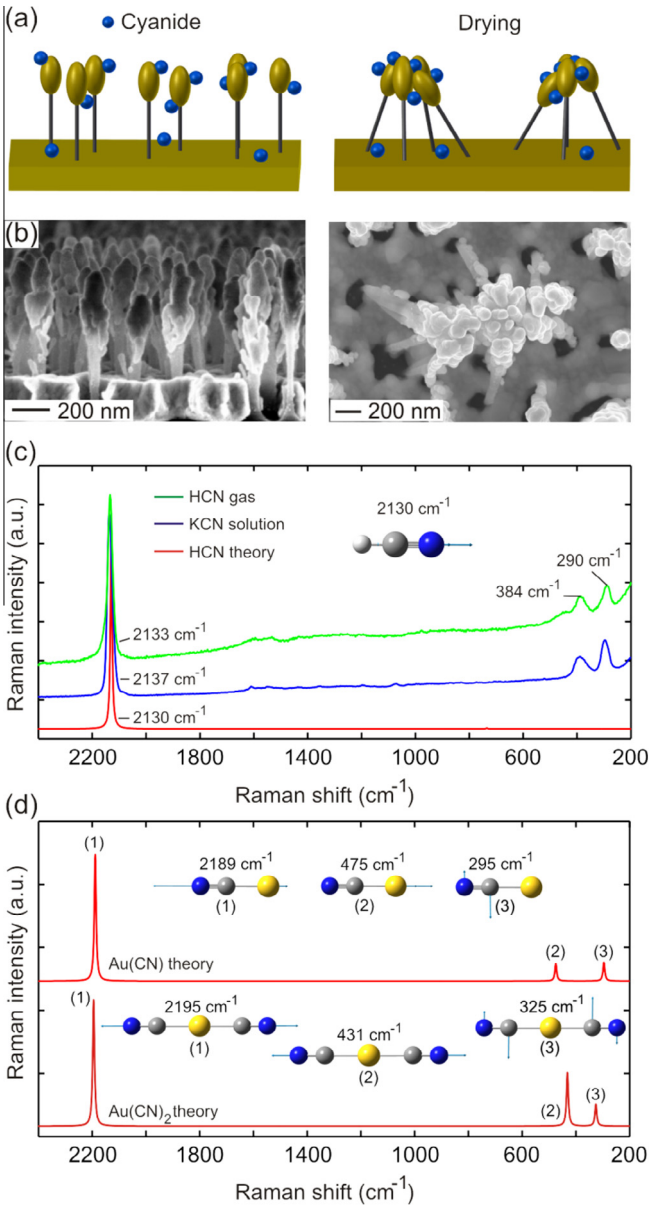

Fig. 1. (a) Illustration of the leaning of nanopillars. When immersed in liquid and subsequently dried, capillary forces make the pillars lean against each other, creating SERS "hot spots" for enhancement of the Raman signal. (b) SEM images of the Au-coated Si SERS substrate, before and after leaning (Courtesy of Kaiyu Wu). (c) SERS spectra of HCN(g) and KCN(aq) on the Au SERS substrate and theoretical Raman spectrum of HCN. (d) Theoretical Au(CN) and $Au(CN)_2^-$ Raman spectra.

The measurements were conducted in a DXR dispersive SmartRaman spectrometer with thermoelectric CCD cooling, an optical microscope and a 780 nm excitation laser from Thermo Fisher Scientific. An optical microscope was coupled to a single grating spectrometer with 5 cm^{-1} FWHM spectral resolution and ±2 wavenumber accuracy. Two times 5 s scan time at a laser power of 5.0 mW was applied through a 25 μm slit using a 10× magnification lens. Raman wavenumber shifts were collected in the region 100–3400 cm^{-1}. In each experiment all SERS substrates were prepared from the same wafer. In order to avoid cross contamination of the sensitive substrates, samples with different KCN concentrations were left to dry separately and kept in small Petri dishes for transport and storage. One substrate was prepared for each concentration and 10 points were measured for each SERS substrate. In the HCN gas experiments each SERS substrate was

exposed to different HCN gas flow conditions. The SERS signal was then recorded from 10 random points from each SERS substrate. Therefore, the data points in Figs. 2(b), 4(b) and 5(b) show the SERS signal intensity variations (cyanide peaks) measured for a given experimental condition from 10 different measurement points from a single SERS chip. Reference SERS signals were recorded using water droplets prior to KCN or HCN exposures. In all cases a weak Raman band close to 2120 cm^{-1} that is likely due to carbonaceous species on the Au surface is observed [17].

2.2. Gas setup

Five ppm HCN(g) in N_2 from the supplier AGA (Linde AG, Pullach, Germany) was used for cyanide gas exposure. Four ppm was the lowest stabile concentration the supplier could guarantee. The setup consists of an open flow cell connected with a pressure controlled gas inlet, see insert in Fig. 2(a). The meter to the right indicates the amount of gas left in the tank while the barometer to the left shows the pressure within the system. The large regulator is for gas flow regulation, and the small one is for opening and closing the gas flow. Thus, when the gas flow is increased, the pressure inside the system rises which can be read on the barometer.

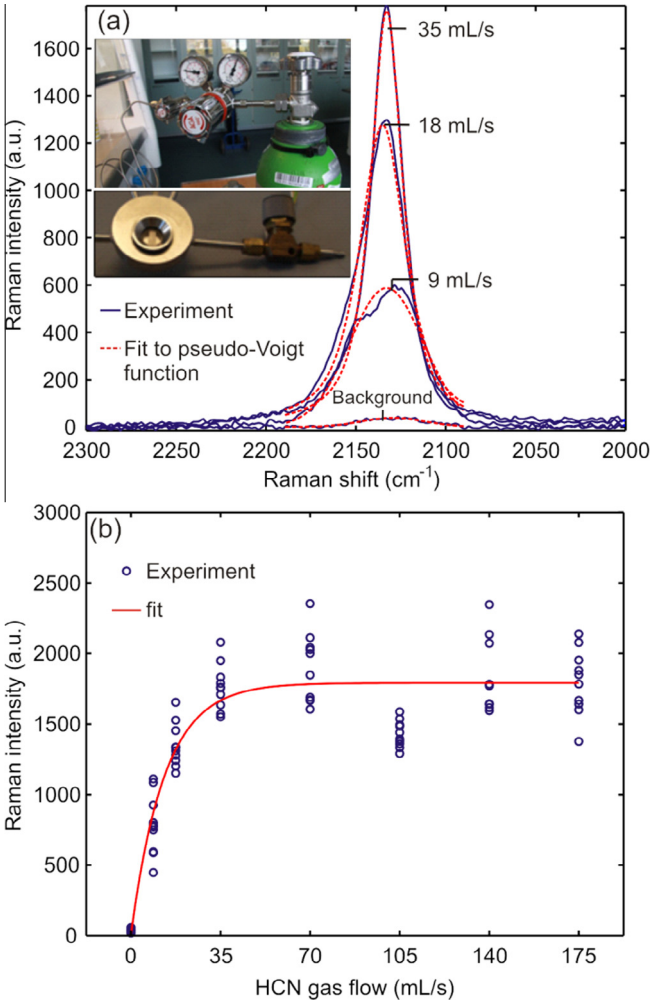

Fig. 2. a (insertion) Setup for 5 ppm HCN gas exposure, including the flow cell in the insertion below. (a) Representative Raman spectra of the pre-leaned SERS substrates exposed to 30 s 5 ppm HCN(g) at 35; 18; and 9 mL/s flow, 30 min. after exposure. The C≡N stretching band at 2134 cm^{-1} is clear and distinct, increasing with increasing flow. (b) 2134 cm^{-1} peak intensity at various gas flows. Above 35 mL/s the signal saturates, corresponding to a gas concentration of 5 ppm.

Via a simple setup using an upturned measuring cylinder in a water bath it was shown that 0.2 bar in the system corresponds to 35 mL/s gas flow. The gas flow is expected to correlate with HCN(g) concentration at low flow-rate. After the regulator, the gas passes through a steel tube to the flow cell placed in the fume hood (bottom insert in Fig. 2(a)). The SERS substrate was placed on a platform inside the flow cell. Prior to gas exposure the SERS substrate was cleaned by 3 min immersion in very pure ethanol (Absolute grade, CHROMASOLV®, Sigma–Aldrich) followed by 3 min rinsing in water (Molecular Biology Reagent grade, Sigma–Aldrich) and left to dry on a clean tissue. The drying caused the nanopillars to lean against each other, see Fig. 1(a) and (b). The cell was purged with the relevant flow for 2 min before the SERS substrate was placed in the flow cell and exposed to the gas for 30 s. In all cases the flow was changed in a random order not to confound the results by sequence of exposure. The gas setup was used for verification of gas detection on the SERS substrate.

2.3. KCN solutions

Aqueous cyanide solutions of potassium cyanide (KCN) were prepared as a model system for precise control of the concentration of cyanide in solution. According to Henry's law either the temperature should be kept low or pH should be high in order to keep cyanide in the solution, preventing it from evaporating as HCN(g) [18]. It would not be feasible to maintain a low temperature, and the approach with pH adjustment was therefore adopted. KCN solutions from 10 nM to 1 mM were prepared and their pH was adjusted to 11 by the addition of NaOH. The SERS substrates were cleaned by 3 min immersion into ethanol (Absolute grade, CHROMASOLV®, Sigma–Aldrich), followed by 3 min in water (Molecular Biology Reagent grade, Sigma–Aldrich). From there they were immersed directly into the KCN solution for 3 min before drying on a clean tissue, making the nanopillars lean against each other, ready for measurements. Due to strong gold-cyanide bonding all cyanide on the substrate is expected to be captured on the nanopillars while the water evaporates. Water with added NaOH to pH 11 was used as reference.

2.4. Data analysis

Theoretical Raman spectra in vacuum were calculated using Gaussian 09W, methods DFT/B3LYP/6-311G and DFT/B3LYP/LanL2DZ, for HCN and Au(CN)x compounds, respectively (no scaling applied).

Each spectrum was jointly fitted and baseline corrected using Voigt profile(s) to fit peaks and a line to adjust for the baseline [23]. The fitting was done in the local area near peaks so that the linear baseline was appropriate. The fitting was carried out using Metropolis–Hastings [13]. Either a mixture of two Voigt profiles or a single Voigt profile was utilized depending on the number of peaks. In the cases where two Voigt profiles were jointly fitted each peak was constrained to be centered ±5 from the desired location. The intensity of each Voigt profile was then used as the intensity response.

3. Results and discussion

3.1. HCN gas experiments

In Fig. 1(c) and (d) the 2133 cm^{-1} vibration is observed in both gas (HCN) and liquid (KCN) SERS experiments. The HCN theoretical vibrational spectrum can explain the origin of the 2133 cm^{-1} mode and corresponds to stretching of C≡N, see insert in Fig. 1(c). In order to understand the origin of experimentally seen vibrations

at 290 and 384 cm^{-1}, additional calculations involving Au-cyanide complexes were performed. We find that Au(CN) and $Au(CN)_2$ display qualitatively similar vibrational spectra, Fig. 1(d). Results show that both 290 and 384 cm^{-1} modes originate from HCN interaction with the Au metal surface, as previously reported by Senapati et al. [26]. This is in accordance with the Elsner equation where the aurat (I) ion complex forms when cyanide is added to gold in the presence of air:

$$4Au + 8CN^- + O_2 + 2H_2O \rightarrow 4[Au(CN)_2]^- + 4OH^-$$

If base is added, cyanide will stay in the CN^- configuration.

In Fig. 2 the SERS spectra of substrates exposed to different flows of 5 ppm HCN(g) are shown. The spectra are recorded 30 min after gas exposure. At lower flows (35; 18; and 9 mL/s) the flow change is reflected in the SERS intensity of the C≡N stretching band at 2133 cm^{-1} (Fig. 2(a)). This indicates that some quantitation is possible at low HCN gas flows. At higher flows (70; 105; 140 and 175 mL/s, Fig. 2(b)) the SERS intensity seems to saturate. Part of the explanation could be that above 35 mL/s most HCN molecules are being "flushed" past the substrate and out of the flow cell, not leaving much time for Au—CN interaction to occur; and therefore only a fraction of the HCN molecules get in actual contact with the SERS substrate. After 4 h the samples were re-measured, and the C≡N stretching band had started to shift +50 wavenumbers. Fig. 3 compares the 35 mL/s gas samples after (a) 30 min, (b) 4 h, and (c) 9 days. It seems as if some dynamics have taken place in favor of the more stable $Au(CN)_2^-$ (aurat (I) ion) complex, in Fig. 3 represented by the extra peak at 2186 cm^{-1}. In total, four bands occurred, at 2133 cm^{-1} and 2186 cm^{-1}, respectively representing the pure C≡N stretching mode of CN^- and the $Au(CN)_2^-$ complex [6,26], and also the Au-C stretching mode at 384 cm^{-1} and the Au-CN bending mode at 290 cm^{-1} are present [26]. The results in Fig. 3 are much similar to those obtained by Premasiri et al. [20] who measured SERS spectra of 0.25–1 ppm NaCN on an Au sol–gel. They report a +50 cm^{-1} shift of the C—N stretching band from low to high CN concentration. We believe the reason for this shift can be that the higher concentrations of cyanide have had sufficient time for dissociation of the Au, forming the mentioned dimer complex. If they had repeated the measurements later either the shift would have been more pronounced or the cyanide would have evaporated as HCN(g). According to Senapati et al. [26] it took about 60 min for a cyanide ion concentration of 800 ppb to dissociate their Au nanoparticles completely, whereas for 800 ppt it would take 180 min. Therefore, at high concentrations the dimer complex forms at a higher rate. The samples of our study were measured again after 9 days, and now the C≡N stretching peak is completely shifted to the 2186 cm^{-1} mode (Fig. 4). It seems as if there is a preference towards the more stable $Au(CN)_2^-$ complex, which increases during storage. This again is in accordance with the results obtained by Senapati et al. [26]. At the same time the Au—CN bending mode at 290 cm^{-1} decreases, probably due to a restricted space for this type of movement in the dimer case. It should be mentioned that the reason why the substrate becomes saturated at a relatively low Raman intensity could be that many hot spots are occupied due to pre-leaning of the nanopillars.

3.2. KCN serial dilution

Since it is difficult to precisely control low concentrations of cyanide in the gas phase, aqueous solutions of KCN were prepared to carry out serial dilution experiments, mimicking lower HCN concentrations in a controllable manner and producing quantitative SERS. KCN is a water soluble powder, and since it is the CN part which is interesting to Raman experiments, KCN in solution works as an optimal model system for precise analysis. To test the correlation between CN concentration and CN Raman signal, KCN solutions from 10 nM to 1 mM were prepared.

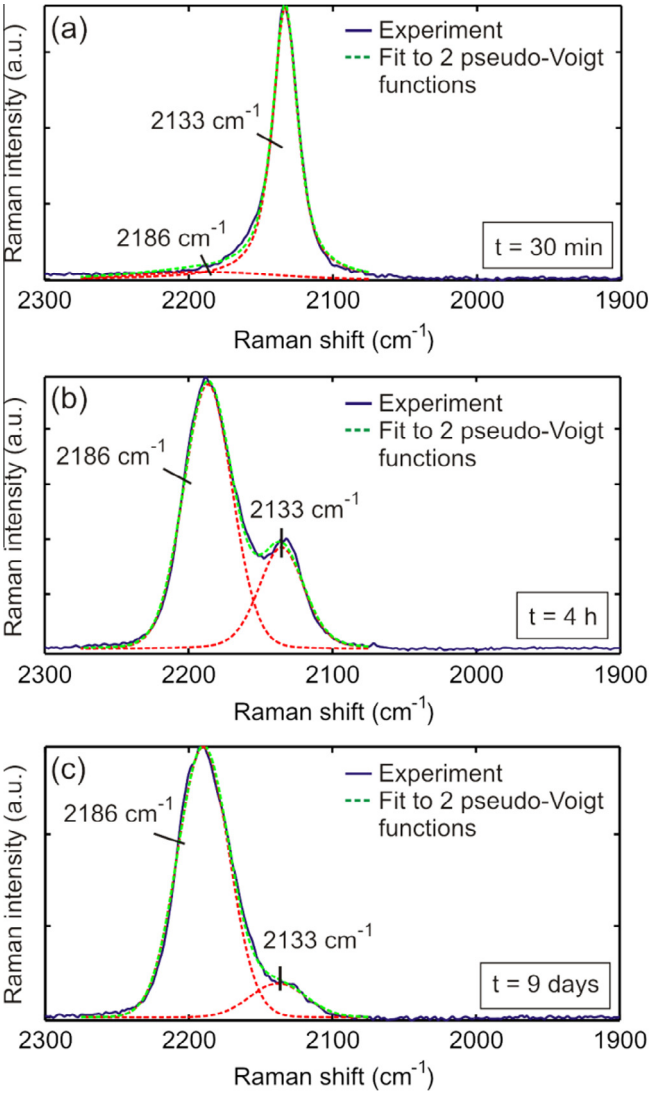

Fig. 3. Representative SERS spectra of 35 mL/s 5 ppm HCN(g) samples measured after 30 min; 4 h; and 9 days. The rearrangement of cyanide from the CN (2133 cm^{-1}) to the $Au(CN)_2^-$ (2186 cm^{-1}) configuration is clearly seen. (Not to scale.)

Representative SERS spectra of the KCN serial solution experiments are presented in Fig. 5(a). The stretching peak of cyanide's triple bond is clear and distinct at ~2137 cm^{-1} where it decreases with decreasing cyanide concentration. As reference, the background spectrum of water (with added NaOH to pH 11) is included for comparison. In Fig. 5(b) the intensity of the stretching peak of C≡N is plotted as a function of KCN concentration. A linear correlation is seen from 100 nM to 1 mM, although it is difficult to distinguish between 100 nM, 10 nM and water. In attempt to distinguish 100 nM from 10 nM KCN and reference samples, principal component analyses (PCAs) including all three cyanide related vibration bands (~2137, 384 and 290 cm^{-1}) were performed, see detailed description in supplementary information. The results indicate that the observed difference between 100 nM and 10 nM KCN concentrations can be largely attributed to signal background fluctuations. However, the results showed nearly a linear relationship between the KCN concentration and the first Principal Component in the 10^{-5}–10^{-7} M KCN concentration range, see Fig. S.6 in supplementary information. This is similar to results

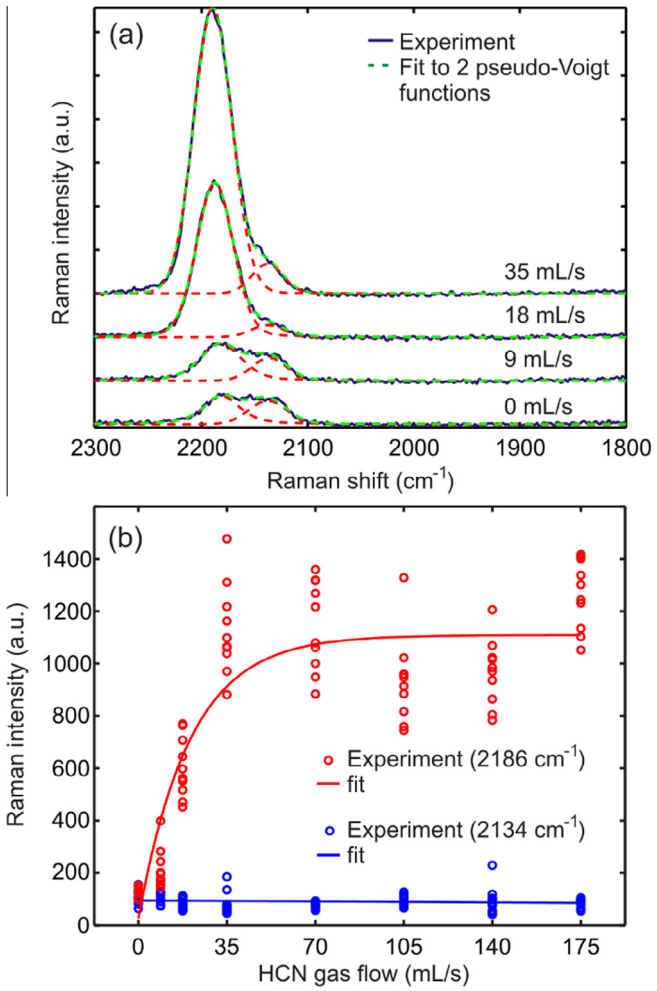

Fig. 4. (a) Representative Raman spectra of the HCN(g) exposed SERS substrates re-measured after 9 days. It is seen that the C≡N stretching mode has shifted to the 2186 cm^{-1} $Au(CN)_2^-$ state. (b) (Blue) 2134 cm^{-1} peak intensity at various gas flows, re-measured after 9 days. There is not much left of the CN monomer. (Red) 2186 cm^{-1} peak intensity at various gas flows, after 9 days. Based on the mean response, Pearson correlation of the intensity of the peaks in Figs. Fig. 2(b) and 4(b) is 0.92. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reported by Thygesen et al. [28] where only the C≡N mode at ~2137 cm^{-1} was utilized in the data analysis. In the latter work, the upper detection limit for a linear relationship between the C≡N band signal intensity and KCN concentration utilizing Au colloids was close to 10^{-5} M while the lower limit was around 10^{-6}–10^{-7} M.

According to Enderby et al. [9] the median value of HCN in the breath of CF children with *P. aeruginosa* lung colonization is 13.5 ppb, which corresponds to 0.75 μM in solution. Explanation is given in the following. Take for instance a 0.05 mL drop of 1 μM KCN weighing approximately 0.05 g. This will contain:

$$0.05\ \mathrm{g}/(18.00\ \mathrm{g/mol}) = 2.8 * 10^{-3}\ mol\ H_2O \text{ and}$$

$$(1 * 10^{-6}\ \mathrm{mol/L}) * (0.05 * 10^{-3}\ \mathrm{L}) = 5.0 * 10^{-11}\ mol\ KCN$$

$$n_{KCN}/n_{H_2O} = (5.0 * 10^{-11})/(2.8 * 10^{-3}) = 18 * 10^{-9} = 18\ ppb.$$

13.5 ppb/18 ppb = 0.75. So, when quantitative SERS applies, the SERS intensity of 0.75 μM cyanide in solution is expected to correspond to the median intensity of the breath of a CF child with PA lung colonization. Breath concentrations for the present application are thus expected to correspond to aqueous cyanide concentrations in the range between 100 nM and 1 μM.

As seen in Fig. 2(b), ~2000 cps seems to be the point of saturation for cyanide in gas phase. The level is similar to 100 μM KCN(aq), which with the above calculations would give 1.8 ppm and not 5 ppm as was the case. This is no surprise as the Au nanopillars used for gas detection were pre-leaned prior to cyanide exposure. Therefore fewer molecules could be "caught" in the hot spots between nanopillars than with cyanide in solution. By letting the alkaline KCN solution dry, all cyanide on the substrate will get in contact with the Au nanopillars, optimizing the Au—CN interaction, including the interaction inside the forming hot spots. It is well-known that pre-leaning of the pillars leads to a lower signal than leaning post-exposure [24]. This means the signal might be expected to be 2–3 times lower with breath than with cyanide in solution; but preliminary tests have shown that the vapor in people's breath is sufficient to make the pillars lean, making pre-leaning unnecessary.

In the serial dilution experiments the C≡N stretching band also shifted during storage, but only when pH had not been adjusted to 11. Like with gas the shift was about 50 cm^{-1} towards higher wavenumbers. Premasiri et al. [20] measured on NaCN(aq) without adjusting the pH. This way Au could dissociate and form the $Au(CN)_2^-$ complex, which probably has led to the shift of the

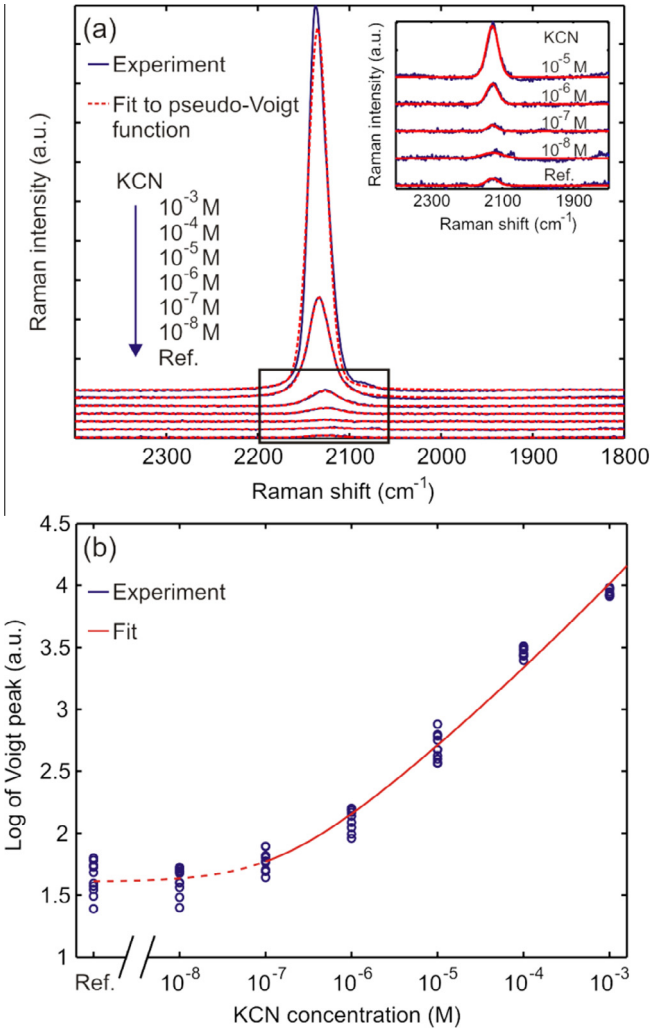

Fig. 5. (a) Representative SERS spectra of KCN concentrations from 10^{-8} to 10^{-3} M and the reference solvent. (b) Raman intensity of the cyanide stretching peak around 2140 cm^{-1} as a function of potassium cyanide concentration. Note log scales on both axes.

C≡N stretching band. When pH is kept high as in the present case, Au will most likely stay as Au^0 and not oxidize to form the aurat (I) ion.

4. Conclusion

By use of the applied SERS technique we have shown that it is possible to quantify the amount of cyanide down to ppb level, which is needed for detection of *P. aeruginosa* lung colonization in the breath of children with cystic fibrosis.

It was possible to distinguish samples with different KCN concentration down to 1 μM (corresponding to 18 ppb) using the C≡N stretching region located close to 2133 cm^{-1}, thus the detection limit was between 18 ppb (detected) and 1.8 ppb (not detected). Future work includes measurements on bacterial cultures and patient samples.

Conflict of interest

The authors declared that there are no known conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sbsr.2015.07.002.

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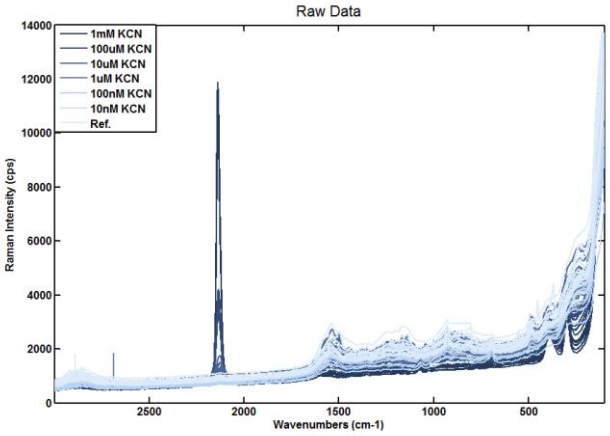
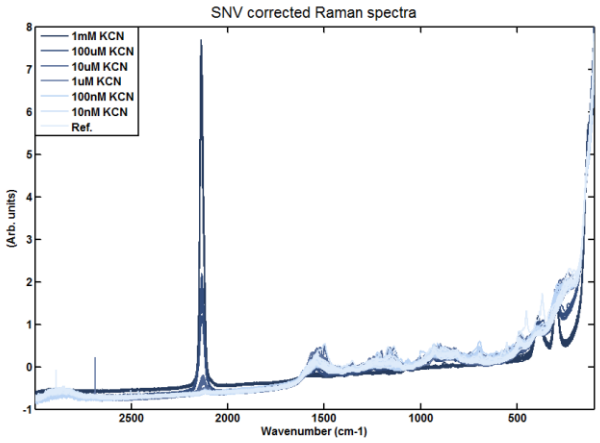
Supplementary information

SERS data on the KCN and water samples were analyzed by Principal Component Analysis (PCA), using MATLAB R2014a (The MathWork, Inc.) equipped with the PLS Toolbox 782 (Eigenvector Research, Inc.). PCA is a variance-based method looking for variations in the dataset (Wold et al., 1987). The direction of largest variance is denoted the first Principal Component (PC1). PC2 is perpendicular to PC1, explaining the direction of second largest variance, etc. PCA can be used to explore groupings and differences among samples, and to find the variables (wavenumbers) or regions of variables where interesting variations occur. Various preprocessing methods were investigated, and the best PCA used SNV transformation followed by mean-centering (MC). Prior to applying PCA, the spectra were normalized using standard normal variates (SNV) and mean-centering (MC). SNV is a method for transforming the spectra in order to minimize the intra-measurement variation resulting in more orderly arranged spectra (Barnes et al., 1989). MC is a simple deduction of the average spectrum applied to the data in the current model, enabling samples to be presented around the origin of the score plot. In order to avoid over-fit of the models, segmented cross-validation (CV) was applied using random subsets with 10 data splits and 5 iterations. In CV some of the samples are left out during the calculations and then included for model validation. Fig S.1 is the raw SERS data on KCN serial dilution samples, while Fig S.2 is the data after applying the standard normal variates (SNV) transformation (Barnes et al., 1989). Fig S.3 is the preprocessed data after SNV and mean centering (MC). The figures show how the unwanted baseline effects found in the raw spectra are removed by SNV and what the data looks like when mean centering has been applied. The preprocessed 1 mM KCN spectrum almost comprises the first loading of the PCA in Fig S.4 (b).

S.1)



S.2)



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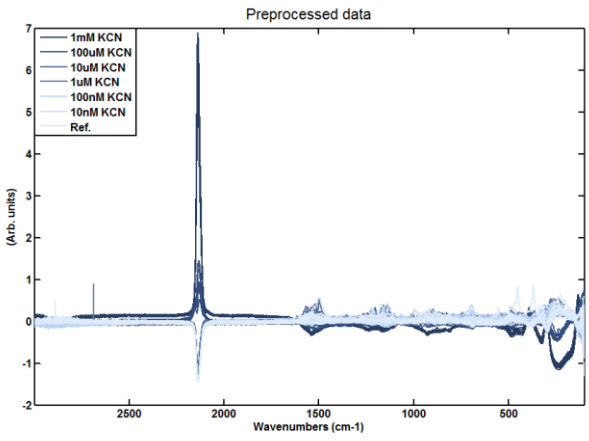


Figure S.1) Raw SERS data on KCN solutions and reference solution. **S.2**) SERS data after SNV transformation. **S.3)** Data after preprocessing (standard normal variate followed by mean centering). It is seen that the high concentration KCN spectra comprise the main variance.

Principal component analysis on regions 200-450 and 2000-2250 cm^{-1}

In Fig 5b a univariate analysis based on one single wavenumber out of the entire spectrum is presented. This approach is intuitive and straightforward to use, but it can be critical to base results on only one measured band intensity e.g. it is very sensitive to interferences and outliers. A way to overcome this, and to make a more robust analysis, is by including a higher number of variables. By a multivariate approach more information may be obtained as even very small spectral changes can be discovered. Based on the C≡N stretching band alone, quantitation was not possible on the 10 and 100 nM KCN and water samples. Therefore PCAs were initially performed on the entire dataset to gain knowledge about wavenumbers of significance. Fig S.4 presents a stepwise analysis on the KCN data. From the score plot (PC1 vs. PC2) in Fig S.4 (a) it is clear that the samples with the two highest KCN concentrations completely dominate PC1 because they contribute most to the variance in data. At the same time those are the only ones that can be distinguished, as opposed to the 50 samples inside the circle. The corresponding loading plot in Fig S.4 (b) shows that the stretching peak of cyanide (2000-2250 cm^{-1}) and the region for Au-C stretching and Au-CN bending from 200-450 cm^{-1} contribute most to this variance. In order to try to improve the separation, a PCA was made including only the 50 samples, which could not be distinguished in Fig S.4 (a), and only the spectral regions; 200-450 cm^{-1} and 2000-2250 cm^{-1}. The score plot in Fig S.4 (c) shows a better spread of the samples which in PC2 vs. PC3 fall into groups according to their KCN concentrations. The 10 µM KCN samples are seen in the upper right region of the score plot, with high scores on both PC2 and PC3. To the opposite, with negative PC2 and PC3 scores, the 10 nM and reference samples are found. Between the mentioned groups the samples with “intermediate” KCN concentrations are located. The loading plot in Fig S.4 (d) shows that the 10 µM KCN samples have high C≡N stretching intensity, while all samples with high PC2 scores have high Au-CN bending intensity. Samples with negative PC2 and PC3 scores have more of the 260 cm^{-1} band and less of C≡N and Au-CN. It is well-known that samples in a score plot lying opposite of each other, on a straight line through the plot’s origin, will have opposite properties according to the loadings, which goes well along with the observations seen in the PCAs and the knowledge we have about the samples. Finally, a PCA was made on the three groups that could not be separated by a univariate approach, i.e. only the 10 and 100 nM KCN and the reference samples. The score plot in Fig S.4 (e) shows that it is possible to distinguish the groups with SERS, and Fig S.4 (f) shows that this is mainly due to the Au-CN band at 290 cm^{-1} and the Au-O band at 232 cm^{-1}. Au-CN is more intense in the 100 nM samples (with high PC1 scores), and Au-O (attributed to water) is more pronounced in the 10 nM and reference samples with negative PC1 scores. PC3 is slightly noisy and should not be ascribed too much significance.

S.4)

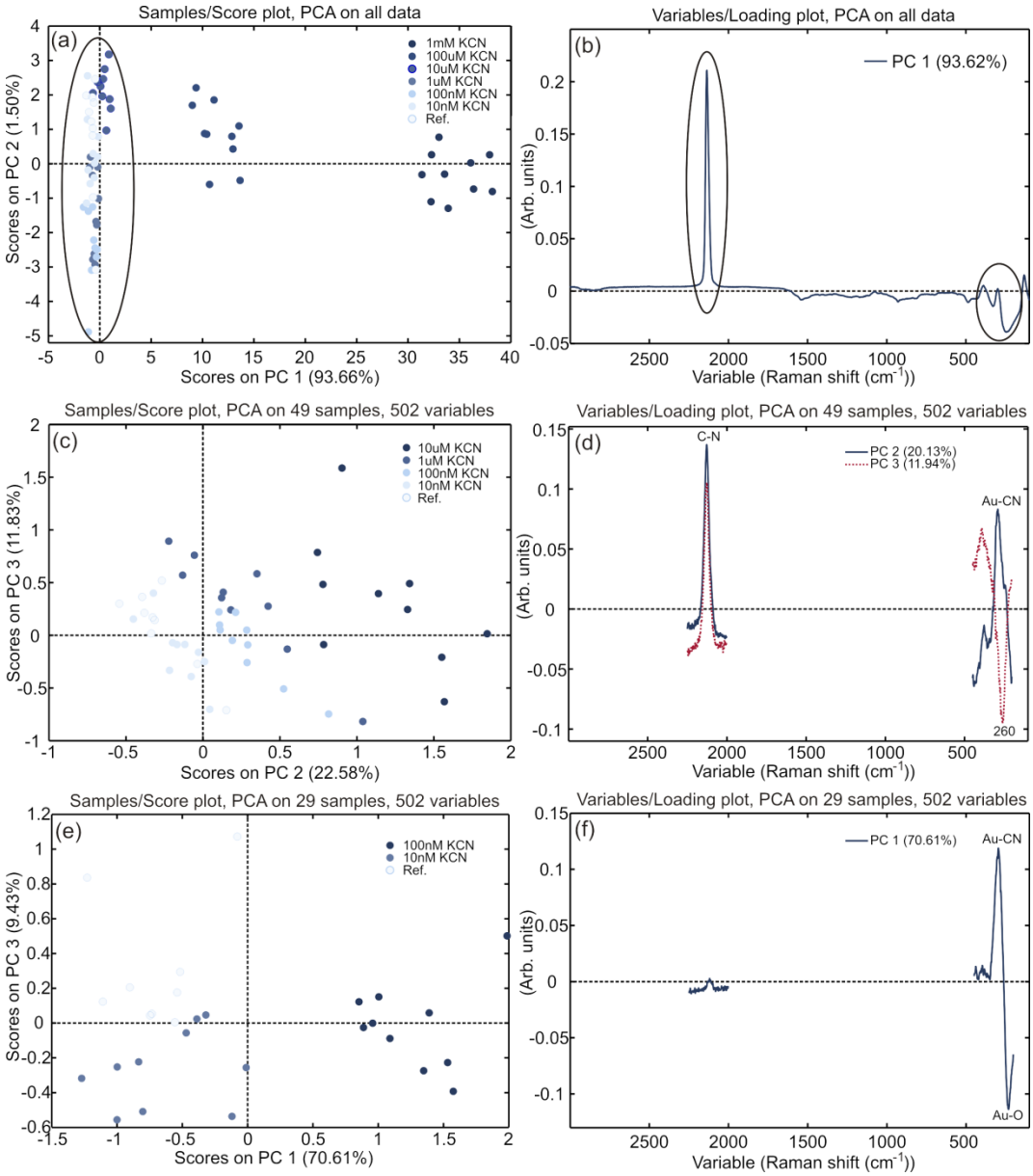


Figure S.4: a) PCA score plot (PC1 vs. PC2) of all KCN data: 70 samples and 3008 variables (wavenumbers). As expected, 1 mM and 100 µM KCN samples completely dominate the plot in the direction of PC1 explaining 93.66% of the entire variation in the dataset. **b**) Loading plot of PC1 corresponding to the previous score plot. It is seen that the spectral regions 2000-2250 and 200-450 cm^{-1} contribute most to the variation explained by this PC. **c)** Score plot of a PCA on only the 50 samples inside the circle of a) and the 502 variables (wavenumbers) from inside the circles of b), PC2 vs. PC3. High concentration KCN samples are found in the upper right part of the plot, opposite the low KCN concentration and reference samples. **d)** Loading plot corresponding to the score plot in c). It is seen that samples with high PC2 and 3 scores have high concentrations of the C≡N stretching and Au-CN bending modes, whereas samples with negative PC3 scores are dominated by the 260 cm^{-1} band. **e)** Score plot of PCA on only 10 and 100 nM KCN and reference samples. PC1, describing 70.61% of the variance, separates 100 nM KCN samples from the 10 nM and the reference samples which are separated in the direction of PC3. **f)** Corresponding loading plot, showing that samples with high PC1 scores have high Au-CN, whereas negative PC1 scores correspond to higher Au-O modes. A small peak is also seen at 2120 cm^{-1} which is not dominant in these samples.

In Fig S.5 a zoom is presented on the averaged KCN spectra. It is seen that the Au-CN band at 290 cm^{-1} is very difficult to determine for concentrations below 10^{-4} M.

S.5)

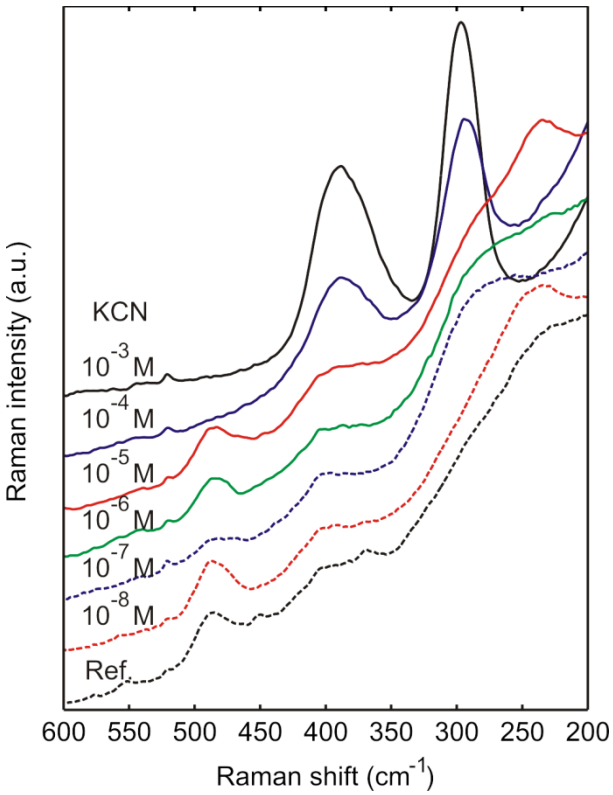


Figure S.5: Zoom on the low end KCN raw data, average of 10 measurements. The Au-CN band at 290 cm^{-1} is clear in the 10^{-3} and 10^{-4} M spectra after which it is difficult to determine due to strong fluorescence and interference with neighboring peaks.

Therefore caution must be taken when interpreting the results from the PCA. It looked like it was possible to distinguish samples with different KCN concentration down to 100 nM (corresponding to 1.8 ppb) by use of the C≡N stretching region and the region for cyanide and water attached to gold. Furthermore, plotting all KCN samples onto PC1 of Fig S.4 (f) does not give a straight line, and thus the PCA is inconclusive.

S.6)

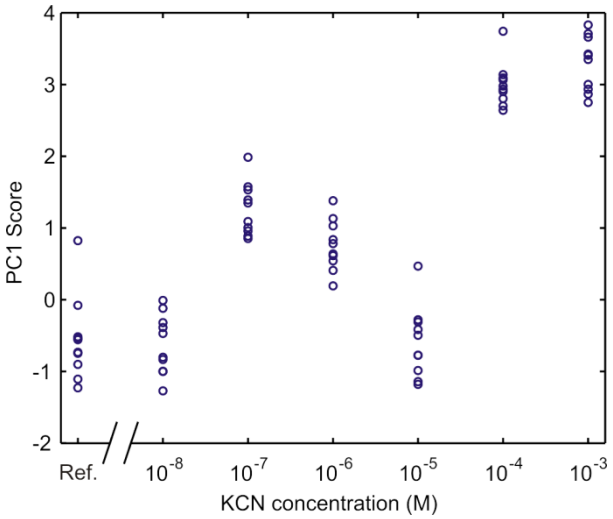
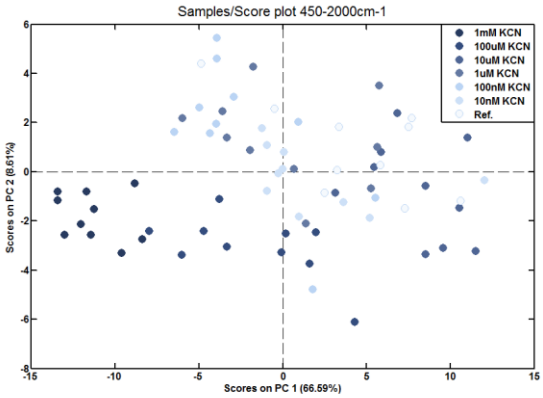


Figure S.6: The scores for the entire KCN concentration data set (1 mM – 10 nM) when explained using PC1 from Figure S.4(f).

Principal component analysis on the complementary region 450-2000 cm^{-1}

A PCA was made on the spectral region between the regions used in the data analysis in Fig S.4. Fig S.7 shows the scoreplot and Fig S.8 the loadingplot of the initial PCA made on all samples and all variables from 450-2000 cm^{-1}. One millimolar and 100 µM samples are mainly seen in the lower left region of the scoreplot (with negative PC1 and PC2). The remaining samples are scattered. The loadings are noisy.

S.7)



S.8)

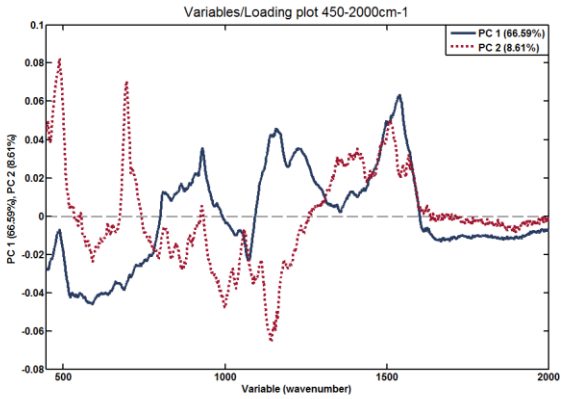
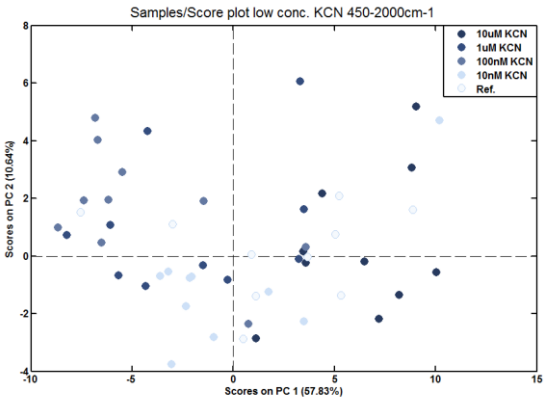


Figure S.7) PCA scoreplot of all samples in the SERS region 450-2000 cm^{-1}, preprocessed by SNV and MC. **S.8)** Loadingplot corresponding to the scoreplot in S.7.

In the raw and preprocessed spectra this region presented a more ordered nature for higher concentration KCN samples than for low. (S.1-3). The reason is a reduced shot noise, leading to a lower level of noise in the spectra, which is why the samples turn out grouped in the scoreplot. Because the samples with highest KCN concentrations could almost be grouped, another PCA was made on the samples with KCN concentrations from 10µM and below. The resulting scoreplot is scattered, and the loadings are even noisier than before. This means that the applied spectral region has a limited significance when it comes to KCN quantification.

S.9)



S.10)

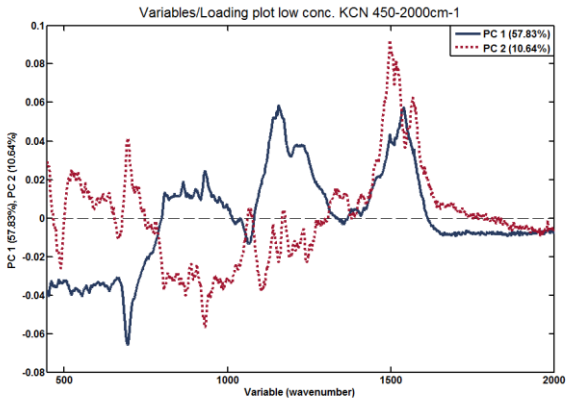


Figure S.9) PCA scoreplot of all samples with KCN concentrations of 10 nM – 10 µM and reference samples. **S.10)** Loadingplot corresponding to the scoreplot in S.9.

Paper II

”SERS detection of the biomarker hydrogen cyanide from *Pseudomonas aeruginosa* cultures isolated from cystic fibrosis patients”

In review with Scientific Reports.

Title

SERS detection of the biomarker hydrogen cyanide from *Pseudomonas aeruginosa* cultures isolated from cystic fibrosis patients

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Keywords: Cystic fibrosis (CF), *Pseudomonas aeruginosa*, Hydrogen cyanide (HCN), Surface-enhanced Raman spectroscopy (SERS), Gas phase detection

ABSTRACT

Pseudomonas aeruginosa is the primary cause of chronic airway infections in cystic fibrosis (CF) patients. Current methods for *P. aeruginosa* identification are invasive, and often there are too few bacteria in the sample to culture. Therefore persistence is seen from the first *P. aeruginosa* culture in about 75% of young CF patients, and it is important to discover new ways to detect *P. aeruginosa* at an earlier stage. The *P. aeruginosa* biomarker hydrogen cyanide (HCN) contains a triple bond, which is utilized in this study because of the resulting characteristic C≡N peak at 2135 cm^{-1} in a Raman spectrum. The Raman signal was enhanced by surface-enhanced Raman spectroscopy (SERS) on a Au-coated SERS substrate. After long-term infection, a mutation in the patho-adaptive *lasR* gene can alter the expression of HCN, which is why it is sometimes not possible to detect HCN in the breath of chronically infected patients. Twelve clinical strains isolated from CF patients were evaluated, and HCN was clearly detected from overnight cultures of all wild type-like isolates. After *lasR* mutation in later isolates from the same patients, HCN could only be detected where the *lasR* mutation had taken place at the 5' terminal (upstream) of the gene.

Abstract word count: 200.

INTRODUCTION

Individuals with cystic fibrosis (CF) have an inherited defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, causing thickened, dehydrated mucus to form on mucociliary surfaces. This in turn increases the risk of e.g. airway infections. *P. aeruginosa* lung infections are the largest threat to the wellbeing and survival of CF patients, and it has been shown for a cohort of young Danish CF patients that an estimated 75 % have a persistent infection already from the first detection of *P. aeruginosa*[1].

If the child is unable to expectorate, today's methods consist of either a larynx swab, which is insensitive, or induced sputum (naso-laryngeal suction), which is extremely invasive. *P. aeruginosa* can be difficult to detect, especially in children and young adults without a chronic infection because they have few bacteria in their airways. Therefore more sensitive and non-invasive methods are being investigated utilizing the fact that HCN is a biomarker for *P. aeruginosa*[2,3,4]. In nature, *P. aeruginosa* emits the poisonous gas hydrogen cyanide (HCN) in order to kill competitive microorganisms. This wildtype (WT)-like behavior is also expected to occur in the lungs, at least during the initial stages of infection. To sustain a persistent colonisation and infection, adaptation is necessary. In many cases this involves mutations of different genes, so-called "patho-adaptive" genes. One of the genes that have been suggested as being "patho-adaptive" (i.e. beneficial for *P. aeruginosa* to mutate) is *lasR*[5,6]. The *lasR* gene is a transcriptional regulator necessary for HCN production[7]. A mutation in the *lasR* gene can imply a downregulation of the HCN production. According to the clinical CF program in Denmark, patients are seen once a month in the outpatient clinic and deliver a sputum sample coughed up from the lungs. Depending on the patient's age and ability this is either done by expectoration or, as in most young patients, induced by a tube inserted through the nose into the larynx to provoke a cough reflex after which a sample is obtained through the tube (naso-laryngeal suction). Sputum samples are then cultured to identify pathogenic microorganisms.

Several groups have investigated HCN production by *P. aeruginosa* in vitro[3,8,9]. The drawbacks of all the applied methods are that they need extensive sample handling before measurements can be obtained, and that it can only be done in solution. Gilchrist and co-workers are investigating the possibility of detecting HCN in the breath of CF children, collecting breath samples for investigation by selected ion flow tube – mass spectroscopy (SIFT-MS), which is an expensive and complex procedure[10]. An optimal sensor would be a cheap and simple point-of-care device, allowing for measurements to be carried out directly on human breath, without pre-handling of the samples.

Raman spectroscopy is a fingerprinting technique that measures the inelastic scattering of monochromatic light when interacting with matter. The frequency shift (Raman shift) of the inelastically scattered light corresponds to the energies of the molecular vibrations[11]. HCN contains a triple bond between C and N, which will result in a Raman band around 2135 wavenumbers (cm^{-1}). But since the inelastically scattered light is only a tiny part of light scattered from a sample ($<10^{-8}$), Raman spectroscopy in general has a weak signal-to-noise ratio, and there is a need for signal enhancement. Using surface-enhanced Raman spectroscopy (SERS) the Raman signal of small molecules is enhanced by utilizing the collective oscillations of conducting electrons (surface plasmons) that take place in the vicinity of clustered noble-metal nanoparticles[12,13]. SERS has been used in a wide array of medical applications[14,15,16]. An example hereof is using label free Ag colloid SERS to detect single-nucleotide mismatch in short DNA sequences to discover even small nucleotide changes[17]. Our SERS substrate has Si nanopillars with Au caps[18] which lean against each other for SERS detection of cyanide in liquid or gas phase, as described previously[19]. In this study we investigate the capability of our sensor to detect the biomarker HCN(g) in emissions from clinical early and late *P. aeruginosa* isolates, to determine its applicability as a non-invasive diagnostic tool for detection of *P. aeruginosa*.

MATERIALS AND METHODS

Preparation of the SERS substrate

The disposable silicon (Si) SERS substrate was etched in a single-side polished Si wafer using 3 min Reactive Ion (plasma) Etching with alternating SF_6 bombardment and O_2 protection of the emerging nanopillars, followed by a 1 min O_2 cleaning step. Before use, Au was evaporated onto the Si nanopillars, forming 225 nm caps used for SERS detection. See Fig 1(a). On the day of exposure the wafer was cut into 6x6 mm chips using a diamond cutter and tweezers. The chips were cleaned by immersion into ethanol (Absolute grade, CHROMASOLV R, Sigma-Aldrich) for 3 min followed by H_2O (Molecular Biology Reagent grade, Sigma-Aldrich) for 3 min and left to dry on a tissue whereby the pillars would lean to enable SERS detection. For easy handling and transportation, the SERS substrate was mounted inside a small Petri dish, using double sided adhesive tape (Scotch), with a Post-It on top, the tacky side facing up for holding the substrate (Fig 1(b, 4). The wall of the Petri dish protected the delicate SERS substrate so it would not get in contact with the bag and be scratched (See section on SERS substrate exposure to bacterial emissions).

P. aeruginosa reference strains

PAO1 is a reference strain commonly used to benchmark various *P. aeruginosa* strains against. PA-SD2 is a PAO1 isolate with a knockout mutation in *lasR*, as described in ref. 20. DK02 is a *P. aeruginosa* lineage that spread among Danish CF patients for over 40 years. The earliest isolate is called DK02-1973, and its *lasR* mutated strain DK02-1979[20].

The clinical strain collection

At the CF clinic at Rigshospitalet in Copenhagen a special collection of bacterial strains has been sampled from CF children and young adults since 2004, resulting in a unique collection for genomics and other evolutional studies to be made[5,21]. From this collection early (*lasR* WT) and late (*lasR* mutant) isolates from five patients have been selected, encompassing three different

clone types, for comparison of adaptations between and within patients. The selected strains originate from CF patients who today are all chronically infected, and the late isolates represent various locations of *lasR* mutations. Selected strains include the first *P. aeruginosa* isolate and at least one later isolate from the same patient after onset of chronicity and with detected mutations in the *lasR* gene.

Ethics

The local ethics committee at the Capital Region of Denmark Region Hovedstaden approved the use of the samples: registration number H-4-2015-FSP. All patients have given informed consent. For patients below 18 years of age, informed consent was obtained from their parents. The study was carried out in accordance with the approved guidelines and the University Hospital Rigshospitalet approved the experimental protocol.

Identification of mutations in *lasR*.

Using a modified version of the pipeline previously described in Andersen et al. (2015)[22], we identified mutations in the CF isolates using the sequences of isolates from young CF patients, previously published by Marvig et al. (2015)[5]. Briefly: Reads were mapped to the *lasR* reference sequence (NP_250121.1) from the reference strain *Pseudomonas aeruginosa* PAO1, using the Burrows-Wheeler alignment tool[23] (BWA version 0.7.12), with the paired-end reads setting. Alignments were filtered to remove unmapped reads, sorted, and indexed using SAMtools[24]. Each isolate was assigned to a read group using Picard Tools[25] (version 1.14) and differences between isolates and the reference *lasR* sequence were identified with SAMtools. Mutations were manually checked using the Integrative Genomics Viewer[26] (IGV, version 2.3.68)**.** The functional impact (missense/nonsense/silent) was determined using the translated sequence of *lasR* in the IGV. The full pipeline can be found in Supplementary material.

Preparation of bacterial cultures

P. aeruginosa strain PAO1, its engineered *lasR* mutant PA-SD2 and two isolates from the classic clinical lineage DK02 from 1973 and 1979[27], as well as pairs of strains from five pediatric CF patients were stored as pure cultures at -80°C, wherefrom they were streaked onto LB agar plates and incubated at 37°C over night. One colony was then inoculated in 10 mL LB growth medium (4 % salt concentration) and incubated shaking at an angle of ~45°, 225 rpm overnight. One mL of the ON culture was added to 9 mL of fresh LB in a 100 mL Erlenmeyer flask, plugged with a two-holed rubber plug, covered with aluminum foil and placed in a 37°C water bath at ~200 rpm, wherefrom SERS substrates were exposed (Fig. 1(b, 6)). One flask was prepared for each measurement needed.

SERS substrate exposure to bacterial emissions

In the setup we used an SK 224-PCMTX4 Universal air sampling pump (SKC Inc., PA, US), connected via a silicone tube to a large Vac-U-Chamber, 231-939 (SKC Inc., PA, US), Fig 1(b, 1-2). Inside the vacuum chamber a 10 L reusable 60 µm PTFE (Teflon) sample bag (Scentroid, ON, Canada), equipped with a 1/2" compression fitting was connected to the sample inlet (3). The bag was specially designed with one end open for mounting the SERS substrate, and closed by a clamp. The sample inlet of the vacuum chamber was connected via a silicone tube to the 100 mL Erlenmeyer culture flask (5), plugged with a rubber plug with two holes, one for the tube and one for air inlet. The pump was operating at ~500 mL/min for 15 min to fill the bag, followed by 5 min holding time for further substrate exposure. Before pumping out bacterial gases, all fittings were tested by closing the vacuum chamber and turning on the pump to let in air from the lab to verify that the bag would inflate and no emissions would be lost when the bacterial culture was connected to the system.

OD and SERS measurements

The optical density (OD) was measured at 600 nm in a UV-1800 spectrophotometer (SHIMADZU, Kyoto, Japan), giving the absorbance as a measure of cell density. SERS measurements were

recorded using a FT-Raman instrument (Bruker VERTEX 70, Bruker Optik, Ettlingen, Germany), equipped with a 1064 nm laser and an InGaAs detector. Samples were measured in a 180 degrees backscattering geometry using 32 scans at a resolution of 4 cm^{-1}. Each sample was measured at 5 different positions on the substrate and the 5 spectra were averaged before subsequent data analysis.

RESULTS AND DISCUSSION

SERS on PAO1 and DK02 ON culture emissions

The DK02 strains were growing much slower than PAO1, and ON cultures were prepared for SERS exposure and measurements. In Fig 2(a-b) it is seen that PAO1 and the isolate from 1973 both emitted substantial amounts of HCN, as seen in the intense C≡N stretching peak at 2135 cm^{-1}, whereas the *lasR* mutated PAO1 and the late (1979) isolate did not. This was as expected since the late DK2 and the *lasR* mutated PAO1 should not have a functional LasR protein.

HCN emission from PAO1 during growth

As seen in the growth curve of Fig 3(a), PAO1 has a lag phase of about 2 hours before onset of exponential growth, with stationary growth, and expected HCN production, beginning after 4-6 hours. According to previous studies HCN production starts at the end of exponential / beginning of stationary phase[8,28]. This is due to the fact that HCN production is quorum sensing (QS) dependent. After a while on the substrate much of the C≡N peak had shifted to 2189 cm^{-1} because of sufficient amounts and the fact that it had had some time to interact with the Au-coated SERS substrate, forming the stabile $[Au(CN)_2]^-$ complex, as explained in ref. 19. Fig 3(b) shows the SERS intensity of the cumulated cyanide peaks at 2135 and 2189 cm^{-1} from 2 until 20 h (ON). It is observed that HCN production begins after 4 h growth and is still detectable in the overnight (20 h) culture, whereas the 2 and 3 h samples only had the same background as the LB reference. The results in figure 3(b) and the shape of the HCN emission curve with time are very similar to previous findings[8,29].

Clinical *P. aeruginosa* strains

Isolates from five CF children were measured with the SERS sensor in the developed setup. All early (WT-like) strains isolated from CF patients emitted HCN. In Fig 4(a) the intensity of the 2135 cm^{-1} C≡N stretching band shows that some of the *lasR* mutated strains also emitted HCN. D'Argenio and co-workers described in 2007 how different *lasR* mutations can have a different

effect on the functionality of the LasR protein[30]. Genome sequencing of the *lasR* mutated isolates[5] showed that isolates with *lasR* mutations located towards the 3' terminal of the gene did not produce HCN (Fig 4(b)), probably because a downstream deletion can be a dominant-negative *P. aeruginosa* mutation resulting in altered molecular function[31]. The other *lasR* mutations were at the upstream or central part of the gene, which did not seem to down regulate the expression of HCN. Furthermore, in CF patient A one *lasR* mutation was a nonsense which in most cases stops the protein from functioning, but apparently not in this case where the mutation was located upstream. In the late isolate there is a clear HCN emission, however the mutation is a nonsense in the beginning (5' terminal) of the gene. This type should abolish the functionality of *lasR* completely. The continued production of HCN in this isolate is in contrast to what we see in other isolates, which indicates that it is the 3' terminal that is important for functionality. The continued production of HCN could be explained by mutations or changes in regulation of other genes that are also important for QS and HCN production circumventing a malfunctioning *lasR*.

Clinical control strains

To make sure HCN can be used as a specific marker for *P. aeruginosa* colonisation, we also tested other clinically relevant species. Fig 5 shows examples of two other bacteria colonising the lungs of CF patients, and known to be able to cause chronic infections: *Stenotrophomonas maltophilia*[32] and *Achromobacter xylosoxidans*[33]. Clinical isolates of these control strains were tested in the same setup as *P. aeruginosa*, but no HCN could be detected.

CONCLUSIONS AND OUTLOOK

We have developed a SERS-based sensor for detection of HCN from *P. aeruginosa* cultures, and a setup for exposure of the SERS substrate to bacterial volatiles. The purpose was to see whether the wild type like strains were producing HCN in sufficient amounts for detection by the SERS substrate and to check whether the *lasR* mutated strains were still producing HCN. For the reference strain PAO1 HCN production started at the end of stationary phase and was still detectable in overnight cultures. In all clinical wild type-like strains isolated from the airways of cystic fibrosis patients there was a clear HCN SERS signal proving the principle that we are able to detect gases from early *P. aeruginosa* isolates. After mutation in the *lasR* transcriptional regulator gene, HCN could only be detected from the strains having the mutation located at the 5' terminal or central part of the gene. *P. aeruginosa* strains with the *lasR* mutation located at the 3' terminal end of the gene did not emit detectable HCN, which may explain why HCN cannot be detected in the breath of all patients with a chronic *P. aeruginosa* airway infection.

Our results suggest that it is indeed possible to use SERS detection of HCN as an early indicator of *P. aeruginosa* infection. The method offers the possibility of a simple, non-invasive point-of-care monitoring device.

CONFLICS OF INTEREST

The authors declared that there are no known conflicts of interest.

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335

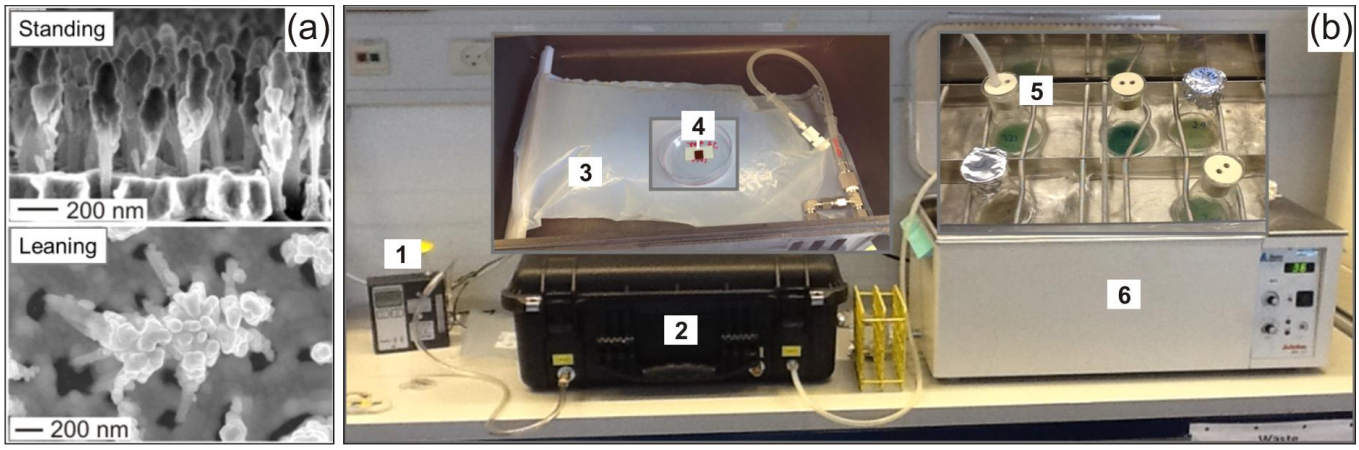
AUTHOR CONTRIBUTIONS STATEMENT

R.L. performed the experiments and wrote the majority of the paper. L.S. wrote the parts on genomics and gave feed-back on the remainder. H.J. identified the bacterial strains, supervised on the experiments and gave feed-back on the paper. T.R. finished the figures. S.M. supervised on the experiments. L.J. gave feed-back on the paper. S.E. supervised on the Raman part and gave feed-back on the paper. A.B. supervised on the experiments and gave feed-back on the paper.

FIGURES

Figure 1

Setup for SERS measurements on bacterial volatiles.

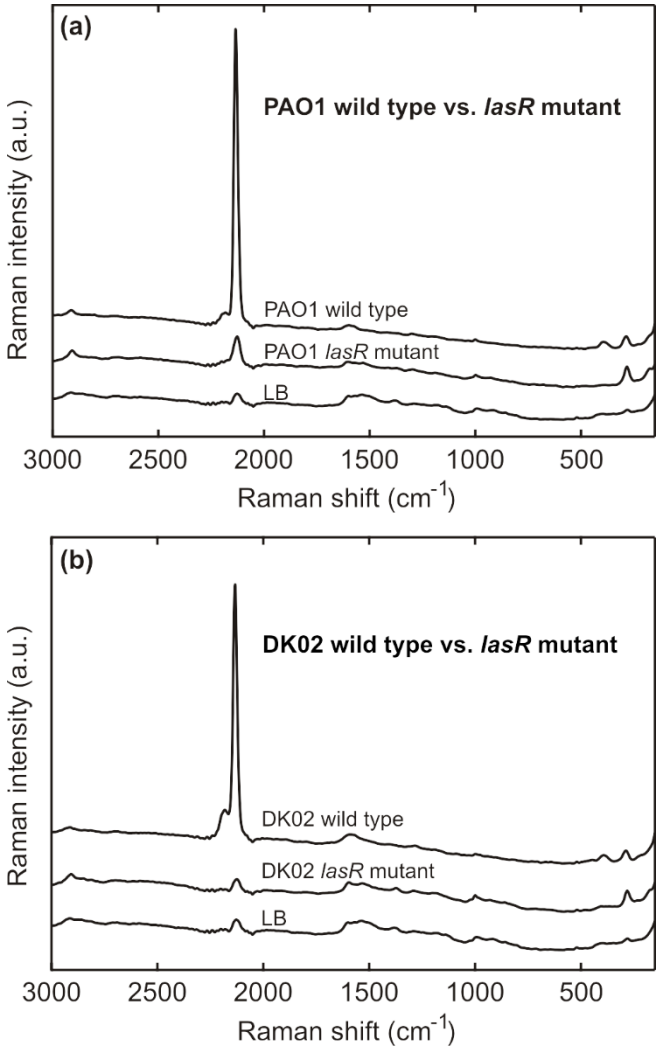


a) SEM images of the SERS substrate before and after leaning for formation of electromagnetic "hot spots" for SERS enhancement. (Courtesy of Kaiyu Wu. Reprinted with permission from editor of ref. 19).

b) The pump (1) induces a vacuum inside the vacuum chamber (2), leading to inflation of the bag (3) and exposure of the SERS substrate (4) to volatiles created by the bacterial culture (5) in the water bath (6).

Figure 2

SERS on overnight cultures of PAO1, the DK02 lineage and their *lasR* mutated strains.

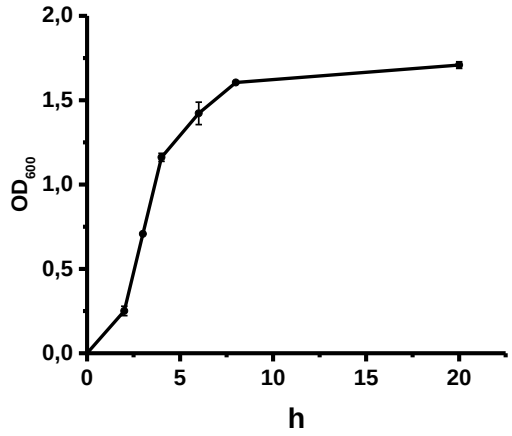


a) SERS on emissions from overnight cultures of *P. aeruginosa* PAO1, wild type vs. its engineered *lasR* mutated strain PA-SD2. b) SERS on emissions from overnight cultures of the *P. aeruginosa* DK02 lineage, wild type from 1973 vs. the *lasR* mutated strain from 1979. Both wild types show intense CN signals at 2135 cm^{-1} and also a small peak at 2189 cm^{-1} due to formation of the $[Au(CN)_2]^-$ complex, whereas the *lasR* mutated strains emit no HCN. Spectra of LB emissions are included as reference.

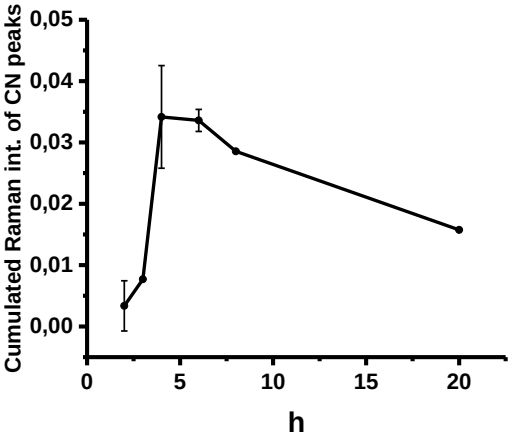
Figure 3

PAO1 HCN emission during growth.

(a)



(b)



a) Optical density (OD_{600}) growth curve for PAO1 in LB medium at 37°C. The lag phase takes about 2 hours, followed by the log phase until about 4-6 hours' growth after which the stationary state occurs. Final OD absorbance is about 1.7 at 600 nm. **b)** Intensity of cumulated cyanide peaks from PAO1 during growth. The cumulated Raman intensity is the (2135 + 2189) cm^{-1} SERS intensity on the PAO1 exposed substrate minus the background signal of the substrate exposed to LB emissions. It is seen that PAO1 starts producing HCN after 4 hours' growth, which is at the end of its exponential / beginning of stationary growth phase. After

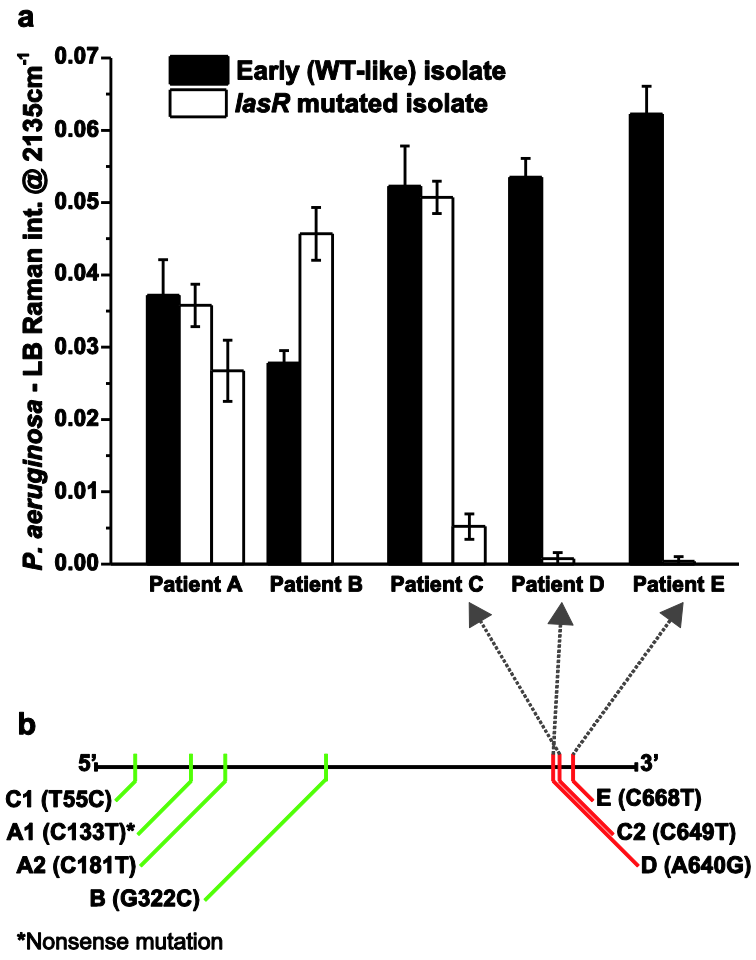
20 hours HCN is still detectable, although not as intense as after 4 hours. The shape of the CN curve resembles previous studies on P. aeruginosa HCN emission during growth[8,28].

382 **Figure 4**

383 **Cyanide production of clinical *P. aeruginosa* overnight cultures from 5 CF patients, as compared to the**

384 **location of *lasR* gene mutations.**

385



386

387

388 a) Cyanide peak intensity of clinical *P. aeruginosa* overnight cultures from 5 CF patients. The peak intensity

389 is the SERS signal at 2135 cm^{-1} minus the background signal of the LB reference samples. It is seen that all

390 WT-like isolates emitted clearly detectable HCN, and so did also some of the *lasR* mutated isolates. For

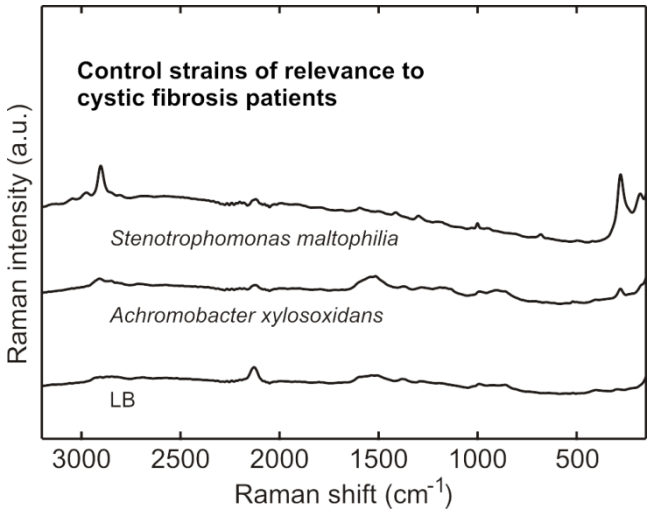
391 patients with two late isolates (Patients A and C), the left white column represents isolate 1 (i.e. A1 or C1)

392 and the right white column represents isolate 2 (i.e. A2 and C2).

b) Map of *lasR* gene mutations in late isolates from the 5 CF patients. It is seen that isolates whose mutations had taken place at the 5' terminal (low base pair numbers) or center part of the gene emitted HCN (high intensity of 2135 cm^{-1} peak in 4(a), indicated with green), whereas isolates with *lasR* mutations at the 3' terminal (high base pair numbers, indicated with red) did not emit HCN (late isolates from patients C, D and E). Letters before parentheses refer to the patient, and the notations in parentheses refer to the position and substitution of base pairs due to the mutation.

Figure 5

SERS on overnight cultures of *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans.*



SERS on emissions from overnight cultures of *S. maltophilia* and *A. xylosoxidans.* None of the bacteria emitted any detectable HCN, as seen in the low intensity of the triple bond peak at 2135 cm^{-1}. Especially the *S. maltophilia* spectrum has different peaks than *P. aeruginosa*. A spectrum of LB emissions is included as reference.

Supplementary material - Title page

SERS detection of the biomarker hydrogen cyanide from *Pseudomonas aeruginosa* cultures isolated from cystic fibrosis patients

Authors

Rikke Kragh Lauridsen, Lea M. Sommer, Helle Krogh Johansen, Tomas Rindzevicius, Søren Molin, Lars Jelsbak, Søren Balling Engelsen, Anja Boisen

Supplementary material

```
#!/bin/bash
ISO=$1 #Input: list of isolate names.

while read line
do

    echo "$line is being aligned with BWA (v.0.7.12) aln paired end function (sampe)"
    bwa aln lasR.fna $line.L*_1.fq.gz > "$line"_read1.sai
    bwa aln lasR.fna $line.L*_2.fq.gz > "$line"_read2.sai
    bwa sampe lasR.fna "$line"_read1.sai "$line"_read2.sai $line.L*_1.fq.gz $line.L*_2.fq.gz > "$line"_aligned.sam

    echo "$line is going through round one in SAMtools v.0.1.18"
    samtools view -Shb -F4 "$line"_aligned.sam > "$line"_aligned.bam
    samtools sort "$line"_aligned.bam "$line"_aligned_sort
    samtools index "$line"_aligned_sort.bam

    echo "$line read groups are added by Picard (v.1.140)"
    java -Xmx2g -jar picard-tools-1.140/picard.jar AddOrReplaceReadGroups INPUT="$line"_aligned_sort.bam OUTPUT="$line"_aligned.RG.bam RGID=CF RGLB=CF_LIB1 RGPL=Illumina RGPU=CF_RG1_UNIT1 RGSM=$line VALIDATION_STRINGENCY=LENIENT

    echo "$line pileups and raw files are being produced by SAMtools v0.1.18"
    samtools mpileup -q 20 -C50 -BDSsugf lasR.fna "$line"_aligned.RG.bam | bcftools view -vg - > "$line"_raw.vcf
done<$ISO
```

Paper III

”Can hydrogen cyanide be detected in breath from children colonised by *Pseudomonas aeruginosa* using surface-enhanced Raman spectroscopy?”

Draft to be completed for submission to Journal of Cystic Fibrosis.

CAN HYDROGEN CYANIDE BE DETECTED IN BREATH FROM CHILDREN COLONISED BY *PSEUDOMONAS AERUGINOSA* USING SURFACE-ENHANCED RAMAN SPECTROSCOPY?

Surface-enhanced Raman spectroscopy to detect early *Ps.a.*

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Abstract

Background: There is an urgent need for a fast and non-invasive tool to detect early *Pseudomonas aeruginosa* airway colonisation in cystic fibrosis patients unable to expectorate.

Methods: Fifty CF children and 19 controls aged 5-17 years were included in the pilot study. A surface-enhanced Raman spectroscopy (SERS) nanochip optimised for detection of trace amounts of the *P. aeruginosa* biomarker hydrogen cyanide (HCN) was mounted inside a Tedlar bag, which the patient breathed into. The SERS chip was then analysed in a Raman spectrometer, investigating the C≡N peak at 2130 cm^{-1} and correlated with sputum cultures.

Results: One new *P. aeruginosa* colonisation occurred during the trial period. The C≡N peak intensity was enhanced in this sample as compared to the subject's 3 other visits. Three additional patients had intense C≡N SERS signals from their breath, but no *P. aeruginosa* was cultured from their sputum. There was a dependency of exposure time.

Keywords: *Pseudomonas aeruginosa*, lung infection, Breath detection, Hydrogen cyanide (HCN), Surface-enhanced Raman spectroscopy (SERS), Pilot study

Abbreviations: SERS: Surface-enhanced Raman spectroscopy; HCN: Hydrogen cyanide; FT: Fourier Transform; CN, C≡N: Cyanide; cm^{-1}: Unit of wave numbers (Raman shift); PCR: Polymerase chain reaction; SEM: Scanning electron microscopy.

1. INTRODUCTION

Pseudomonas aeruginosa airway infections are the major cause of morbidity and mortality in cystic fibrosis (CF) patients [1, 2]. The methods that are being used in the CF clinic to discover *P. aeruginosa* are invasive, and often the sensitivity is limited. It has been estimated that approximately 75% of all newly discovered *P. aeruginosa* infections are already persistently established [3]. Therefore, there is an urgent need for a sensitive, fast, cheap and non-invasive diagnostic tool to detect early *P. aeruginosa* airway colonisation in CF children who are unable to produce sputum or expectorate. *P. aeruginosa* emits the poisonous gas hydrogen cyanide (HCN), which can be an advantage combating other microorganisms in the lungs [4, 5]. HCN is considered a potential *P. aeruginosa* biomarker, and it was detected at median values of 13.5 ppb in children's breath using selected ion flow tube mass spectroscopy (SIFT-MS) [4]. In the SPACE study, Gilchrist and co-workers measured HCN in exhaled breath of CF children, also using SIFT-MS [6]. So far, this methodology has not been optimized to meet the need for fast detection, and measurements were made within 24 hours, whereby some of the exhaled HCN might have been lost. Additionally, chronically infected patients were included, whose *P. aeruginosa* might have ceased producing HCN. Previously we have shown that at some point during adaptation *P. aeruginosa* can stop producing HCN, which can explain why the biomarker cannot be detected in the breath of most CF patients with a chronic airway infection [7].

Between C and N of cyanide there is a triple bond, which gives rise to a distinct peak in a Raman spectrum. Raman spectroscopy is a fast and non-invasive analytical technique extensively used for molecular fingerprinting. The technique identifies analytes by differentiating their molecular vibrational frequencies in the probed Raman scattering signal. However, the limit of detection for traditional Raman spectroscopy is in the range of millimolar concentration [8], impeding the use of the technique for detecting molecules at concentrations below a millimolar. To indicate *P. aeruginosa* infection, HCN below 20 ppb levels needs to be detected in the breath of patients with cystic fibrosis [4]. Surface-enhanced Raman spectroscopy (SERS) may provide a solution. As an advance of Raman spectroscopy, SERS probes the significantly enhanced Raman scattering signal from analytes sitting on the surface of nanostructures made up of noble metals. The enhancement factor can reach 10^7 to 10^8, enabling detection of target molecules at ultra-low concentrations [9]. In addition, the affinity of cyanide towards gold due to the formation of Au-CN complex [10] makes SERS very suitable for detecting trace amount of HCN. For example, colloidal gold nanoparticles have been used to detect HCN in liquid phase at ppt levels in waste water [11]. However, the employed colloidal system lacks the ability to sense HCN in gas phase. Recently, we have demonstrated the fast identification and quantification of gas-phase HCN down to 18 ppb on gold coated silicon nanopillar substrates [12], which are manufactured in large-format by a cost effective process [13]. Our results indicate that SERS on the nanopillar substrate has significant potential towards fast, cheap and non-invasive detection of *P. aeruginosa* lung colonisation from the breath of children with cystic fibrosis. We have also applied the SERS method to detect HCN in emissions from *P. aeruginosa* cultures isolated from CF patients [7]. In this pilot study, we wanted to investigate if the SERS substrate could be used for direct detection of HCN in the breath of CF children with an early *P. aeruginosa* colonisation. To compare *in vitro* results to *in vivo* breath analysis, two chronically infected CF patients were included in the study, which followed 50 CF children for four months at the Copenhagen CF centre of Rigshospitalet. Each month the children

exhaled into 3L Tedlar bags with the SERS substrate mounted inside, which was analysed a few hours later.

2. MATERIALS AND METHODS

2.1 Subjects and study design

Fifty children with CF (median age X years, range 5-17 years) and 19 non-CF, age-matched controls were included in the study. CF patients were excluded if their latest total IgG measured by an Enzyme-Linked-Immosorbent Assay (ELISA) against *Pseudomonas* antigens was above 2.39 ELISA units, or if the number of precipitating antibodies against *P. aeruginosa* was $\geq$2, meaning that they had a chronic *P. aeruginosa* infection [14] The controls were patients and siblings seen in the CF outpatient clinic of Rigshospitalet during the study period, which was January-April 2016. Controls were not included if they had CF, primary cilia dyskinesia (PCD) or asthma. Enrolment took place during the entire study period, and each subject was tested between 1-5 times. Controls were only tested once. SERS measurements were compared with sputum sample, obtained by endo-laryngeal suction or expectoration, and cultured at Department of Clinical Microbiology at Rigshospitalet to investigate if the method was sufficiently sensitive to detect HCN if *P. aeruginosa* was cultured from the patients' airways. The controls only delivered breath and no sputum sample.

2.2 The SERS substrate

The disposable silicon (Si) SERS substrate was etched in a single-side polished Si wafer using 3 min Reactive Ion (plasma) Etching with alternating SF_6 bombardment and O_2 passivation for protection of the emerging nanopillars, followed by a 1 min O_2 cleaning step. Up till 5 days prior to use, Au was evaporated onto the Si nanopillars, forming 225 nm caps used for SERS detection (Figure 1a). On the day of exposure the wafer was cut into 5x10 mm^2 substrates using a diamond cutter and tweezers. The substrates were cleaned by immersion into ethanol (Absolute grade, CHROMASOLV R, Sigma-Aldrich) for 3 min followed by H_2O (Molecular Biology Reagent grade, Sigma-Aldrich) for 3 min and left to dry on a tissue whereby the pillars would lean to enable SERS detection (Figure 1b). For easy handling and transportation the SERS substrate was mounted inside a small Petri dish, using double sided adhesive tape (Scotch), with a Post-It on top, the tacky side facing up for holding the substrate. The wall of the Petri dish protected the delicate SERS substrate so it would not get in contact with the Tedlar bag used for breath collection and be scratched (Figure 1c).

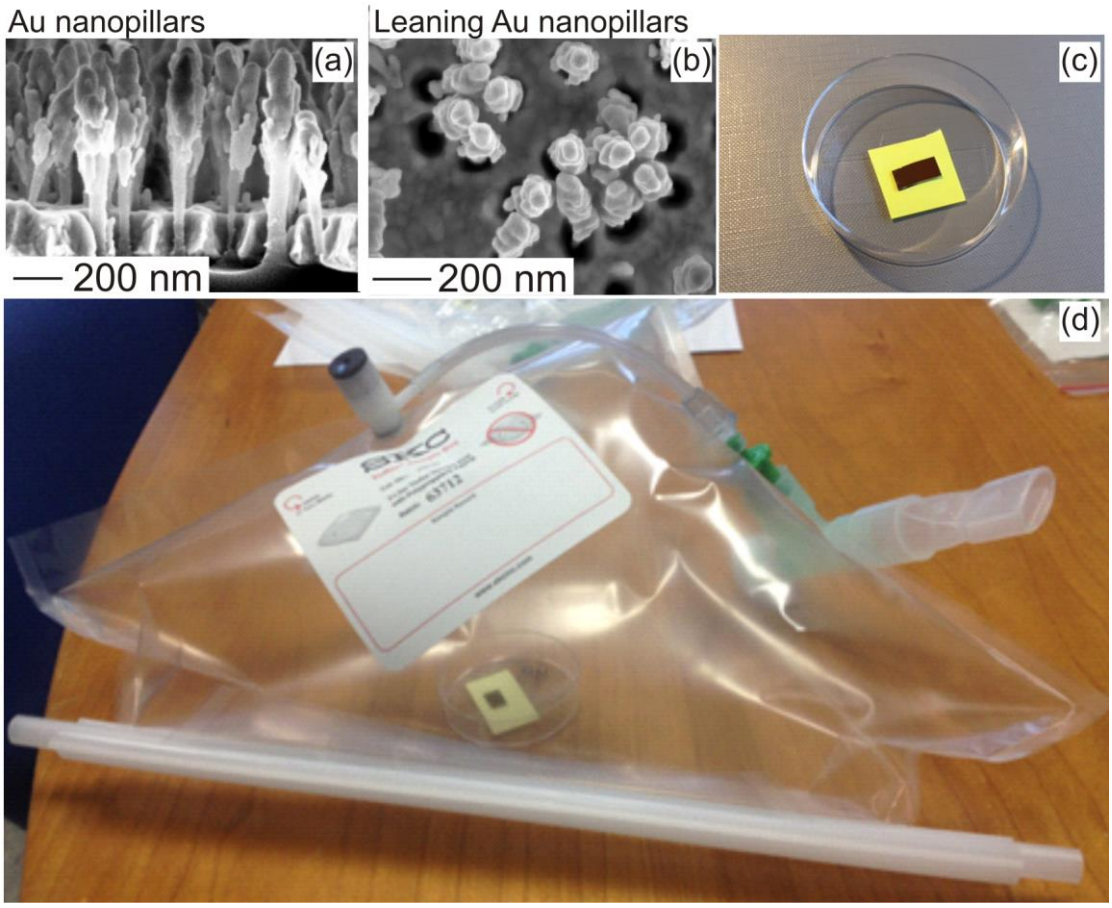

Figure 1: The SERS substrate.
a) Scanning Electron Microscopy (SEM) image of the freely standing nanopillars before leaning.
b) SEM image after leaning of the nanopillars.
c) Petri dish with a gold (Au) SERS substrate mounted inside.
d) The filled collection device with the SERS chip inside, being exposed to exhaled breath. The tip of the PEP flutter is seen to the right. The fitting on top and the clamp at the bottom keep the Tedlar bag closed.

2.3 The collection device

Three L Tedlar sample bags with polypropylene fittings (SKC, Pennsylvania, USA) were used for breath collection, with one large corner cut off, so the Petri dish containing the SERS substrate could be mounted inside (Figure 1d). The bag was closed with a clamp (Scentroid, Ontario, Canada). Part of a disposable latex tube (Philips Respironics, Colorado, USA) was used to connect the inlet of the Tedlar bag to the PEP flutter (Intersurgical, Berkshire, UK) that the child would blow through, equipped with a 3.5 mm resistance (Wellspect Healthcare, Mölndal, Sweden).

2.4 Sample collection

Every child filled the Tedlar bag with 1-5 deep exhalations. The bag was closed and left for 15 minutes before evacuation in a laminar air flow bench. The lid was put on the Petri dish, which was then secured tightly in a small zipper bag for transportation. A short questionnaire was also filled in, regarding present well-being, coffee intake and smoking, because it is known that these stimulants can influence the amount of HCN in the breath [15, 15a].

2.5 Raman measurements

SERS measurements were recorded using an FT-Raman instrument (Bruker VERTEX 70, Bruker Optik, Ettlingen, Germany), equipped with a 1064 nm laser and an indium gallium arsenide (InGaAs) detector. Samples were measured in a 180 degrees backscattering geometry using a laser power of 200 mW with 32 scans at a resolution of 4 cm^{-1}. Each sample was measured at 5 different positions on the substrate.

2.6 Data analysis

The five spectra obtained from different positions on the substrate were averaged in order to increase the signal to noise ratio. The average per sample was subsequently pre-processed using Standard Normal Variate (SNV) [16]. SNV eliminates off-sets and slopes from spectra with similar spectral features aiding visual inspection as well as reducing complexity of later data analysis. Principal component analysis (PCA) [17] was performed using the PLS_toolbox (Ver. 7.9.5, Eigenvector Research, USA) in Matlab 2015a (MathWork, USA) on the pre-processed data both on the full spectral range and a reduced data set with only the HCN peak around 2135 cm^{-1}. In figures, the background was subtracted and curves were shifted up for clarity.

2.7 Ethical approval

The local ethics' committee approved the pilot study, which was assigned the number H-15016027. Informed consent was obtained from the parents or from the children aged 15 years or older.

3. RESULTS

3.1 Demographics

A total of 50 CF patients and 19 controls were included in the pilot study (Table 1). With 4 months and 50 CFs, about 2 new cases of *P. aeruginosa* colonisation were expected (according to the outcome of [6]), but a few patients with chronic infections were included, leaving fewer naïve cases.

Table 1. Demographics of included CF patients and controls

Cystic fibrosis patients		
Median age, years (range)	11 (5-17)	
Gender, number (%)	Males 23 (46)	Females 27 (54)
No. of visits by the patients in total: 87		
Controls		
Median age, years (range)	8 (5-17)	
Gender, number (%)	Males 12 (63)	Females 7 (37)
No. of visits by the controls in total: 19		
Underlying diseases of the controls: Phacomatoses, Secondary pulmonary condition, Cough, Pneumonia, Pulmonary atelectasis, Stridor, Pulmonary hemoptysis, Acute bronchitis.		

3.2 Comparison of *in vitro* and *in vivo* SERS

Previously, *in vitro* SERS measurements were made on *P. aeruginosa* cultures isolated from chronically infected paediatric CF patients in the outpatient clinic [7]. Two of these patients were also included in the pilot study because their isolates represented two different trends in the *in vitro*

study. In the *in vitro* study, all first (wild type-like) isolates emitted detectable HCN. After it had turned into a chronic infection, subject A had a *lasR* mutated *P. aeruginosa* isolate which still produced HCN (Figure 2a), while subject B's *lasR* mutated *P. aeruginosa* isolate had turned off the expression of HCN (Figure 2b). Therefore, it was interesting to investigate whether this trend was also seen *in vivo*. The isolates were obtained five years before, so the bacteria could possibly have mutated further in the meantime. As seen in the figures, no HCN was detected from their breath.

Although chronically infected, patient A only cultured *Stenotrophomonas maltophilia* and no *P. aeruginosa* from sputum, whereas patient B cultured both mucoid and non-mucoid *P. aeruginosa*.

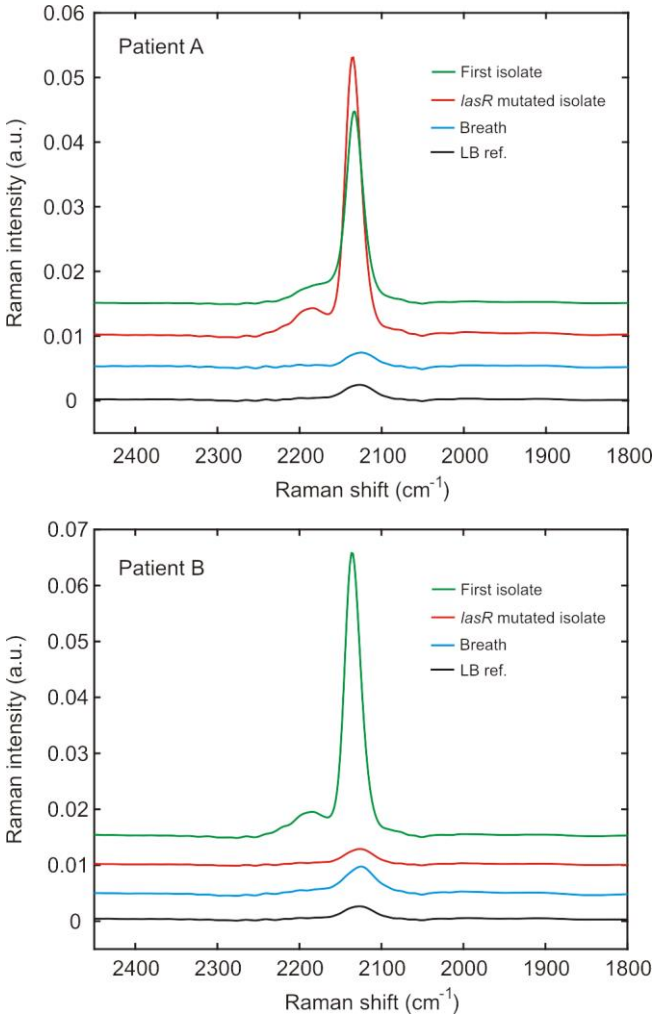

Figure 2: *In vitro* and *in vivo* SERS on *P. aeruginosa* isolates and breath from two CF patients who today are chronically infected. (A) Both the wild type-like (Green) and the *lasR* mutated isolate (Red) emitted HCN, which can be seen from the intense C≡N peak at 2135 cm^{-1}, whereas no HCN was detected from the patients' breath (Blue). (B) The wild type-like isolate emitted HCN, which did neither the *lasR* mutated isolate, nor the breath sample. SERS on headspace of Luria Broth (LB) growth medium is included as reference (Black). There is a background peak in the SERS spectra close to where the cyanide peak is located.

3.3 Four cases of elevated cyanide signal

There was one new case of *P. aeruginosa* during the 4 months' trial period. The subject was a 15 year-old male with a positive sputum culture at his 3rd out of 4 visits. Figure 3 shows the SERS spectra from his four visits, with a more intense triple bond signal about 2130 cm^{-1} from visit no. 3. A zoom on the peak in Figure 4b shows that the peak is slightly shifted towards higher wave numbers, away from the background signal. After antibiotic eradication therapy between the third and fourth visit to the outpatient clinic, the background peak is back to where it started.

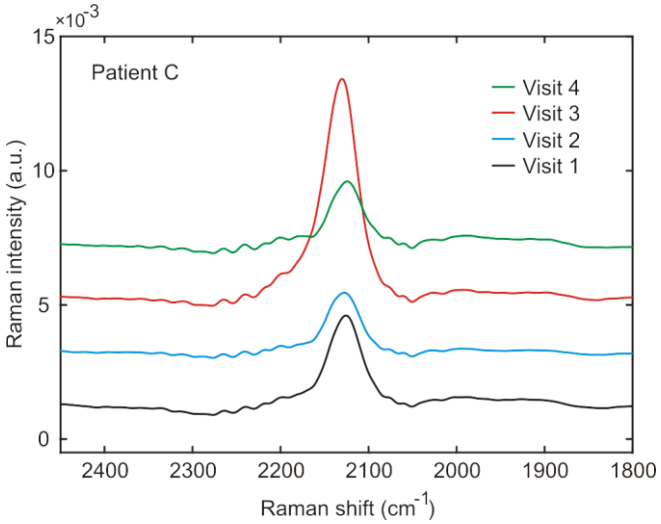

Fig 3: Zoom on the triple bond peak about 2130 cm^{-1} from subject C's visits. The intensity is increased at visit no. 3, and the peak is slightly shifted towards higher wave numbers.

Three additional times during the trial period the SERS triple bond signal was elevated in breath samples,but none of these subjects had *P. aeruginosa* cultured from their sputum. In one of the cases, the Tedlar bag had been left for 60 min before the SERS substrate was taken out, and it turned out that this had also been the case in the 3rd visit of subject C, and it is uncertain if this had an effect. Two of the incidents were from children whose parents were smoking in-door at home, which may have been detected on the chip.

DISCUSSION

We included 50 patients with CF, of which two were known to have a chronic *P. aeruginosa* lung infection, while the rest were completely free, or *P. aeruginosa* naïve. The reason to include two patients with chronic infections was that we wanted to compare our *in vitro* results [7] on their first colonising strain and later *lasR* mutated isolate to *in vivo* breath analysis, because these cannot be expected to be the same [18]. Only one centre was included, to be able to follow the patients closely and register anything that could influence the results.

The pilot study holds significant learnings. The HCN peak height may depend on exposure time, which needs to be further exploited. One approach could be to leave the device for e.g. 60 minutes for complete exposure to occur. In the *in vitro* study 15 min exposure time was applied, which was sufficient, and it is a learning to take to the next clinical trial. Another way to overcome the exposure time issue could be to condensate the breath directly onto the SERS substrate. Disposable devices for this are available (like the R-Tube from MESM Ltd.) [19], but it can be speculated whether patient compliance would be affected by having to breathe into a "chimney"

device like this instead of into the applied “balloon-like” device which most of the children thought was fun. It could also be considered to make the substrate alkaline, so that a maximal amount of the cyanide would be expected to stay on the Au surface (according to Henry’s Law [20]).

Patient A, whose *lasR* mutated *P. aeruginosa* isolate emitted HCN, was 8 years old; and at the time the strain was isolated she was 3. An explanation to why no HCN was detected in the breath of this patient could be that the bacteria have mutated even further since the isolate was cultured back in 2011. Even though this CF patient has about 25 precipitating antibodies against *P. aeruginosa*, no *P. aeruginosa* had been cultured from her last 3 sputum samples. Only when naso-laryngeal suction was performed, mucoid *P. aeruginosa* was cultured; and it can be questioned whether young patients are able to expectorate properly. In the SPACE study, 21 chronically infected patients were included, who had not been cultured positive in their swab or sputum samples for the last 12 months [6], and it can be questioned what the probability would be to culture positive during the trial period. In future studies we will suggest to consider using a more sensitive reference method, such as PCR instead of just sputum culture to detect whether *P. aeruginosa* is present in the sputum sample or not. Another issue regarding bacterial culturing is the transportation conditions of the sputum samples which could be optimized to better secure survival of the microorganisms. Samples should be either cooled down or kept at 37°C and transported immediately to the clinical microbiologists and not wait up till 24 or 48 hours due to internal mail delivery.

In the *P. aeruginosa* case cultured during the trial, the size of the Raman shift was not outside the normal shift seen across patients; however, the patient specific shift (assuming a given patient has an individual peak position) can be an indication. More similar cases are necessary to investigate or reject this hypothesis. Two of the 3 patients with false positive cyanide SERS signal were children whose parents were smoking in-door at home. The third patient had an immediate microscopy result indicating few Gram-negative rods, but when the final microbiological result came, it was *Moraxella catarrhalis*, which is a diplococcus, that can hardly be mistaken for a Gram-negative rod. The question is, whether Gram-negative rods were present in the beginning but not cultured subsequently. Another observation was, that these children can be exposed to passive smoking as they enter the CF centre, which is located on the main floor, where people are standing right outside, smoking, because this is prohibited inside the hospital.

A major drawback of the applied method is the background signal on the Au SERS substrate, which has also been reported elsewhere [21]. It seems to be enhanced by the warm and moist breath. The three cases with intense triple bond signal and negative sputum culture were followed for five months after study termination, and none of them cultured *P. aeruginosa* during the follow-up period, suggesting the increased triple bond peak must have been false positives. Regarding the control subjects, these need to be selected more carefully. Some were too sick, and some might have had *P. aeruginosa* in their lungs, and their sputum was not cultured. Ideally, the study period should have been longer or more patients should have been included to obtain more new positive *P. aeruginosa* cultures. To ease logistics and keep it to one centre, primary ciliary dyskinesia (PCD) patients could have been included, too. The PCA analyses did not show strong trends in the pre-processed spectral data. It is important to remember that the spectral measurements as well as the sputum sample results carry large uncertainties due to exposure time differences, delayed analysis of sputum possibly leading to increased number of false negative results, etc., making it challenging to relate the two.

Due to a limited number of includable patients, only one new case of *P. aeruginosa* was cultured during the trial, and the SERS cyanide signal was increased compared to the patient's three other visits. There was a dependency of exposure time, and it seemed like passive smoking could also be detected from the children's breath.

Conflicts of interest
The authors declared that there are no known conflicts of interest.

Author contributions
RKL planned and carried out the trial; fabricated SERS substrates and assembled the devices; included and instructed subjects; performed Raman measurements and wrote the majority of the paper. HKJ planned the trial regarding in- and exclusion criteria; supervised during the trial period and gave feed-back on the paper. PBS made the data analyses. KW wrote the section explaining SERS and gave feedback on the paper. TR, SM and SBE provided supervision, and TR finalised the figures. KGN helped to establish equipment for breath collection, identify the patients, and had the overall responsibility of the study. AB provided supervision and feedback on the paper.

Acknowledgements
We would like to thank all CF patients and controls and parents who took part in the study. The staff, and especially Majbritt Presfeldt, Maria Charlotte Philipsen and Marianne Skov from the outpatient clinic at the Danish Paediatric Pulmonary Service at Rigshospitalet are acknowledged for their patience and for making this study possible. We would also like acknowledge Michael Stenbæk Schmidt for inventing the first nanopillar SERS substrate, and to thank Flemming Larsen for seeing the opportunity of forming this CF project.

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Supplementary

Flow Chamber for low concentration HCN(g)

Workplace description for exposure of SERS substrates to 5ppm HCN(g)

1

Background

- A sensor is being developed to detect trace amounts of cyanide in the breath of cystic fibrosis patients infected with *Pseudomonas aeruginosa* in the lungs
- In order to verify SERS measurements on liquid cyanide samples, SERS substrates must be exposed to a known concentration of cyanide gas (5ppm)
- For this purpose a tank of 5ppm HCN in N_2 has been ordered

2

Principle

- The SERS substrate is placed inside a glas jar with a specially designed lid with gas inlet and outlet fittings
- The jar is screwed up into the lid thus holding the jar and connecting tubes
- The lid must be equipped with a sealing O ring and swage locks
- The setup has been designet to minimize human exposure to the gas

3

What to do – step by step

- When the gas tank arrives its labels and papers are checked to verify its content to be 5ppm HCN in N_2
- The 5ppm gas tank is attached firmly to the wall so it will not be knocked over in case it is hit
- A flowmeter is attached to the reduction valve of the gas tank

4

What to do – step by step

- A thin (inside diameter ≤ 1mm) steel tube is attached to the gas tank at one end and to the gas inlet of the specially constructed lid through a swage lock
- Inside the glas jar the outlet of this tube ends close to the bottom of the jar to prevent the gas flow from running straight out through the outlet of the jar

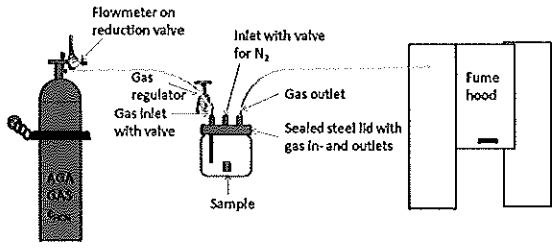
5

What to do – step by step

- Just before entering the jar the gas flow must be adjusted by a gas regulator
- The gas is led out through the gas outlet and into a fume hood
- At the same time an inlet for atmospheric air is opened to replace the gas inside the chamber
- This air inlet is equipped with a vent to prevent the gas inside the chamber to flow out into the laboratory

6

Setup



7

To run an experiment

- The fume hood is turned on
- The gas outlet towards the fume hood is opened
- The gas regulator is closed
- The air inlet is closed
- The sample is placed in the jar which is screwed firmly onto the lid
- The gas inlet is opened
- The flowmeter is turned on

8

To run an experiment

- The gas tank is slowly opened
- The gas regulator is gently turned
- When the desired flow is reached the gas outlet is closed and flow continued for the set time (according to chamber volume and inside diameter of the tube – see calculations)
- The gas tank and regulator are closed
- The sample is left for appropriate exposure time

9

To finish an experiment

- The N_2 inlet is opened
- The gas outlet is opened
- The chamber is purged with N_2
- The N_2 inlet is closed
- The gas outlet is closed
- The jar is screwed off the lid
- The sample is analyzed

10

To be designed

- Steel lid with 2 inlets and 1 outlet
- Hole in side of fume hood
- Connecting thread for flowmeter on reduction valve

11

Calculations

- A 1st order reaction kinetics approach is applied to calculate the concentration of HCN inside the chamber at any given time:

$$\frac{dc_{HCN}}{dt} = \frac{Q_{HCN}}{V_R}\left(c_{HCN,0} - c_{HCN}\right)$$

Q_{HCN} = gas flow rate
V_R = chamber volume

12

Calculations

$$c_{HCN}(t) = (1 - \exp(-\frac{t}{(Q_{HCN}/V_R)})) c_{HCN} \Leftrightarrow$$

$$\boxed{\frac{c_{HCN}(t)}{c_{HCN}} = 1 - \exp(-\frac{t}{(Q_{HCN}/V_R)})}$$

13

Calculations

$$\frac{c_{HCN}(t)}{c_{HCN}} > 0.99 \Rightarrow$$

$$\exp(-\frac{t}{Q_{HCN}/V_R}) < 0.01 \Leftrightarrow$$

$$\frac{t}{Q_{HCN}/V_R} > 2\ln 10 \Leftrightarrow t > \frac{V_R * 2\ln 10}{Q_{HCN}}$$

14

Calculations

$Q_{HCN} = 5$ mL/s

$V_R = 100$ mL

$$t > \frac{100\text{ mL} * 2\ln 10}{5\text{ mL/s}} = 40 * 2.3 \approx 100\text{ s} \approx 2\text{ min.}$$

15

Worst case scenario

In case the gas tank is left open or emptied out at once, 2000L of 5ppm HCN in N_2 will exhaust in ≥ 45m^3 resulting in an immediate HCN concentration of ≤ 0.25ppm which is well below Arbejdstilsynet's hygienic limitation of 5 ppm in which a person is allowed to work for up to 8 hours per day*. With a fume hood flow of ½ m/s all the air has been exchanged within < 3 mins.

*https://arbejdstilsynet.dk/~/media/3FA26655715740ED84EA28EC1191FB62.ashx

16

Chemical risk assessment

Handling and storage of cyanide compounds

Date: _____January 19th 2015_____

Completed by: ___Rikke Kragh Lauridsen_____

Director (signature): _____

Complete the form on the basis of your experience both of the work process/directions and of the relevant safety data sheets.

DETAILS OF THE WORK PROCESS

Firstly, remember always to reduce the handling of cyanide compounds to a minimum.

- To work with cyanide: prepare yourself well, inform the people around you, and have all the items ready before taking the cyanide out of the cabinet.
- Remember to start the emergency suction of the fume hood before cyanide powder is brought into the fume hood.
- Once you have weighed the amount needed, put the powder back into the locked cabinet.

To make a 1.5x10-3M dilution of KCN:

- Remember to put on lab coat, extra length gloves and goggles.
- Make a sign clearly stating: “TOXIC ! CYANIDE” (e.g. on a single-use towel) and place it inside the fume hood.
- Turn on the scales inside the fume hood.
- Calibrate the scales with a 50 mL measuring flask on it.
- Turn on the emergency suction.
- Take out the KCN from the toxic cabinet underneath the fume hood. Close the cabinet and leave it unlocked with the key sitting in the lock.
- Use a single-use spatula to transfer 5 mg KCN into the measuring flask.
- Close the lid of the KCN powder.
- Take the flask out of the scales and put on the lid.
- Drag the gloves off your hands, enclosing the spatula inside the gloves. Do it carefully so the spatula does not penetrate the gloves. Throw the gloves with spatula into the black (toxic) waste bin.
- Wash your hands and put on a new set of gloves.
- Lock the KCN powder into the toxic cabinet.
- Add water to the mark of the measuring bottle, put on the lid and turn 10 times.
- Throw the gloves into the normal bin and wash your hands before putting on a new set of gloves.
- Turn off the emergency suction of the fume hood.

Store the solution well marked in a ventilated cabinet.

CHEMICALS
Potassium cyanide (KCN) Milli-Q water
HAZARDS FROM CHEMICALS
Potassium cyanide is an extremely toxic chemical due to its inhibition of metal-containing enzymes, leading to cessation of cell respiration, and death. It can enter the human body through the integument, which is why skin exposure to cyanide can be lethal. Include only the most hazardous substances – describe their hazardous properties and type of exposure– e.g. toxic if inhaled, carcinogenic when in contact with the skin and flammable. Give the quantities if they differ significantly from normal laboratory patterns.
HAZARDS FROM THE WORK PROCESS
Handling of potassium cyanide must be reduced to a minimum. Exposure to skin, eyes, mouth or nose can be lethal. E.g. laser, vacuum, mixing of chemicals, other equipment – e.g. glass equipment under vacuum with risk of explosion.
RISK OF EXPOSURE
Always working in the fume hood and carefully following these guidelines will reduce the risk of exposure to a minimum. It is also essential to work in a clean, quiet and not too crowded environment while handling this toxic chemical. The addition of NaOH to pH 11 will reduce the risk of inhaling toxic HCN formed from aqueous KCN. Assess the real risk in respect of the work process. The fact that the chemicals are dangerous if inhaled does not necessary mean that there is a risk of inhalation during this work process. Consider where in the work process the relevant risk resides: is it present throughout the process or only in a discrete sub-process.
SUBSTITUTION
None possible. Describe here what attempts and deliberations have been made as to the substitution of hazardous chemicals or work processes. Remember that using small rather than large amounts also counts as substitution.

NECESSARY SAFETY MEASURES	
Ventilation	Fume cupboard: x Local exhaust: LAF bench: Other: Are the items listed necessary throughout the work process or only for certain procedures? Give details: While making serial dilutions of KCN it is essential to work in the fume hood. When using KCN(aq) solutions below 10^{-7}M it can done on the table.
Chemical gloves (Give approx. penetration time if possible)	Which: Nitrile gloves with extra length to fill the gap between the hands and the sleeves of the lab coat. Penetration time: Are the gloves necessary throughout the work process or only for certain procedures? Give details: Nitrile gloves are necessary throughout the entire process since the solutions will be adjusted to pH 11.
Other personal protective equipment	Overalls / protective clothing: No Safety goggles: YES Breathing equipment (state filter): No Special footwear (state type): Closed, firm footwear. Other: _____ Are the items listed necessary throughout the work process or only for certain procedures? Give details: Always
Other safety precautions	Specific sources of heat in the event of risk of fire: No Other: Let the people around you know that you will be working with KCN before you start to reduce the risk of being interrupted unnecessarily.
Special emergency equipment:	Special fire-fighting equipment: No Any antidotes: Other: AMBU mask for ventilation in case of an accident. No mouth-to nose with person intoxicated by cyanide.

Special training or instruction:	Statutory training; specify: Instruction in the use of particularly dangerous equipment; specify: Other: Before starting to work with cyanide ask someone capable of giving you correct instructions to come along the first time.

WHAT TO DO IN THE EVENT OF AN ACCIDENT OR SPILLAGE

- Get the person out in the fresh air.
- Be careful not to get intoxicated yourself.
- Start ventilating the person using the AMBU mask.
- Call 112 and ask for an ambulance. Let them know it is cyanide poisoning

Detail steps to be taken in the event of **relevant** accidents, the clearing up and disposal of spillages, the procedure for information on accidents etc.

WASTE

There is a certain container only for cyanide waste with base placed in the right toxic cupboard under the fume hood. Cyanide waste must be handled with care, of course inside the fume hood, taking all the precautions mentioned above.

Instructions on labelling waste bins – which waste group, UN no., etc. – poss. brief details re subsequent delivery of waste to chemical waste collection point (name).

PREGNANT AND BREASTFEEDING WOMEN

Is the work process/area safe for pregnant or breastfeeding women?

Yes: _____ reasons: _____

No: _____ reasons: _____

PROPOSAL(S) FOR IMPROVING SAFETY:

If people working in the lab would clean up after themselves the better space would make working with cyanide safer.

This item is seen as input into a general Workplace Assessment for the institute/department.

SERS detection of HCN gas over KCN solution.

Chemicals:

- SERS substrates + holder
- Potassium cyanide, KCN (s)
- 0,5 M potassium permanganate, $KMnO_4$ (aq)
- Milli-Q water + Bottle with MQ

Glassware:

- 1 L measuring flask + lid
- 100 mL measuring flask + lid
- 50-100 mL measuring cylinder
- Large Petri dish + lid. One set per exposure time.
- Small Petri dish or lid. One piece per exposure time.

Utensils:

- Small single use spatula
- Pipette with single use tip
- Tweezers
- Fume hood with Emergency suction
- Scales
- Timer
- Sign warning "TOXIC! CYANIDE"
- Gloves
- Goggles
- Fine marker

Procedure:

- Fill Milli-Q water into the 1L measuring flask, leaving at least 100 mL unfilled.
- Turn on the Emergency suction of the fume hood.
- Weigh 5 mg of KCN directly in the 100 mL measuring flask and fill up with Milli-Q water.
- Empty the 100 mL measuring flask into the 1L measuring flask and fill up with Milli-Q water.
- Place the large Petri dish in the fume hood and a "TOXIC! CYANIDE" sign with it.
- Place SERS substrates on top of the backside of the small Petri dish or lid.
- Fill 20 mL of the KCN solution into a measuring cylinder, and from there into the large Petri dish.
- The lid is put on the Petri dish which is left for 2 minutes for equilibrium state to be met.
- Place the small Petri dish with the SERS substrates inside the large Petri dish.
- Put on the lid immediately, and set the timer to minutes' exposure time.
- When finished, take out the small Petri dish from the large one and leave to dry in the fume hood.
- Place the SERS substrates in the holder for transportation to the Raman microscope.
- Add drops of 0.5 M potassium permanganate to the glassware containing KNC solution until red.
- Now the solution can be emptied into the sink in the back of the fume hood.
- Rinse all glassware with Milli-Q water over the sink in the back of the fume hood.
- Turn off the Emergency suction.
- Wash the glassware in the large sink and rinse with Milli-Q water before drying.

Biological APV / Risk Assessment
Biological APV for work with *Pseudomonas aeruginosa*

1) Which organisms are involved?

Pseudomonas aeruginosa PAO1 and clinical isolates of PA.

2) Facts about the organism

If working with other organisms than those mentioned above check http://arbejdstilsynet.dk/da/regler/bekendtgorelser/b/biologiske-agenser-57/bilag-8-klassifikation-af-biologiske-age.aspx to determine biosafety class of the organism(s) you are working with

Refer to http://arbejdsmiljo.ku.dk/bioarbmiljo/biologiskeagenser/ for information about biological APV.

Pseudomonas aeruginosa

All work with *Pseudomonas aeruginosa* must be carried out in a Biological Class 2 lab.

Pseudomonas organisms are flagellated, rod-shaped bacteria that can cause disease in humans and animals. It is a Gram-negative, aerobic or facultative anaerobic bacterium in which case it can use nitrate as electron acceptor. Most human illness is caused by one species, called *Pseudomonas aeruginosa*, and mainly in immuno-suppressed humans and patients with (burn) wounds or cystic fibrosis. In healthy individuals colonization or infection is mainly due to hot tubs or poorly maintained pools. These bacteria are wide-spread in nature, including water and soil, and on medical equipment (like catheters). They can even grow at 42°C and in diesel and jet fuel (Wikipedia). Due to a high rate of resistance and to its ability to form biofilms, *Pseudomonas aeruginosa* can be tricky to eradicate. If a *Pseudomonas aeruginosa* infection is not treated successfully, sepsis and death can occur.

Source: http://www.cdc.gov/hai/organisms/pseudomonas.html

3) Working conditions

Detailed instructions can be found in “Instructions for working, disinfecting and dealing with accidents in gene technology Class I laboratories”.

Most importantly: Work with a high standard of hygiene.

Receipt of organisms:

When we receive a foreign organism, most of them are either freeze-dried, on agar plates or in small tubes, where they grow on solid media.

If the organism is freeze-dried, dissolve it in broth and cultivate it. Afterwards inoculate it in either broth or on solid media.

Freezing of cultures:

Prepare a mixture of the bacterial / yeast culture and glycerol. 1 ml mixture will be frozen in small tubes at

-80 ° C.

Propagation:
A freeze culture or a scrape from a freeze culture is grown in broth or agar plates. We scrape with a plastic spreader. Incubate the culture.

Growth experiments / CFU:
Growth experiments can be done in many ways, but the main features is that the organism inoculates in broth to a certain concentration. After that, the growth is followed by either measuring the OD or CFU calculations. The usual amount is typical 100 µl - 1 ml for each sampling. Work with care and avoid formation of aerosols by gentle pipitting, only vortexing/mixing closed tubes/containers etc.

If you transport organisms between workstations, they should be kept in closed containers, such as bottles or tubes with lids.

If you perform operations not described here, you should fill out the "table of the workflow" and "flow diagram" (see Annex).

All work with these organisms will be performed in a Class 1 GMO laboratory (Biological Class 2 lab).

5) Quantity

Normally we work with volumes from 100 µl to 2 liters of growth culture.

6) Harmful aspects

The mentioned organisms can cause illness if you either get them in the mouth or mucous membranes or if you stick yourself with an infected needle. It's important to avoid formation of aerosols, because there is a risk of infection.

7) Pregnant and lactating

No particular risk.

8) Cystic Fibrosis

Areas where work with *P.aeruginosa* is performed must be indicated with appropriate signs and separated from other laboratories where CF patients work.

9) Safety – (how do you avoid being exposed)

(Refer to the instruction: "Instructions for working, disinfecting and dealing with accidents in

gene technology Class I laboratories").

Protective clothing such as fastened lab coats must be worn whilst working in classified areas. Protective clothing should be taken off and stored in classified laboratories, whenever leaving the room, unless material is being transported between two different classified areas.

To avoid contamination of the water in water baths, a chlorine tablet may be added.

When incubation of cultures is taken place on shakers, it is important not to set too fast rotation to avoid

spillage and formation of aerosols.

It is forbidden to eat, drink, smoke and to store food in the laboratories.

Infected liquids (cultures, washing buffer etc) must not be poured down the sink.

Pipetting by mouth is strongly forbidden.

Staff must wash their hands when temporarily leaving the lab (e.g. for coffee break, lunch or the toilet) and also at the end of the work day.

Pipettes should be mechanical in nature.

Care should be taken to minimize formation of aerosols. For example, centrifugation should be in closed containers, and heated inoculation loops should not be used unless they are cooled first. It is also a good idea not to use the Bunsen burner, especially when you work with *Campylobacter* .

Hypodermic needles and sharp objects should be used with caution.

Pouring of cultures into centrifuge tubes and decanting of supernatant after centrifugation may only take place in classified areas.

The following disinfectants can be used:

Chloramine:	1 chloramine tablet per 5 l distilled water
Sodium hypochlorite:	10 ml sodium hypochlorite (15%) per 5 l distilled water
70 % ethanol:	700 ml. 96 % ethanol adjusted to 960 ml with distilled water

Use granules for absorption of spillages.

Use yellow clinical waste bags for disposal of infected waste. These must be closed properly.

Bacterial cultures, etc.

Liquid cultures:	These should be inactivated with chloramine (1 tablet per l) or chlorine (10 ml per l) for at least 30 min before disposing down the lab sink; alternatively, they may be autoclaved in sealed containers.
Petri dishes (plastic):	These should be disposed of in the yellow bag.
Petri dishes (glass):	These should be collected in autoclave bags, which should then be securely closed and decontaminated by autoclaving.

10) Accidents - (what do you do)

(Refer to the instruction: “Instructions for working, disinfecting and dealing with accidents in

gene technology Class I laboratories”).

Spilled cultures should be absorbed as much as possible using granulates/cloths/paper towels, and the area should then be disinfected. Absorbents are discarded in the yellow bag.

If the spillage occurred down the drain or sink, then the spilled culture should be inactivated using the appropriate disinfectant (usually hypochlorite, but this should not be used in connection with acids).

The area around the accident should be disinfected.

If you work with faeces always wear gloves and perform your work in a fume hood, because you don’t know what kind of organism it contains. When your work with faeces is done decontaminate all your equipment with 70 % ethanol.

If you are unlucky enough to stick yourself with a needle that is contaminated with one of the organisms mentioned above you should contact a doctor. This also applies if you get infected material in your mouth.

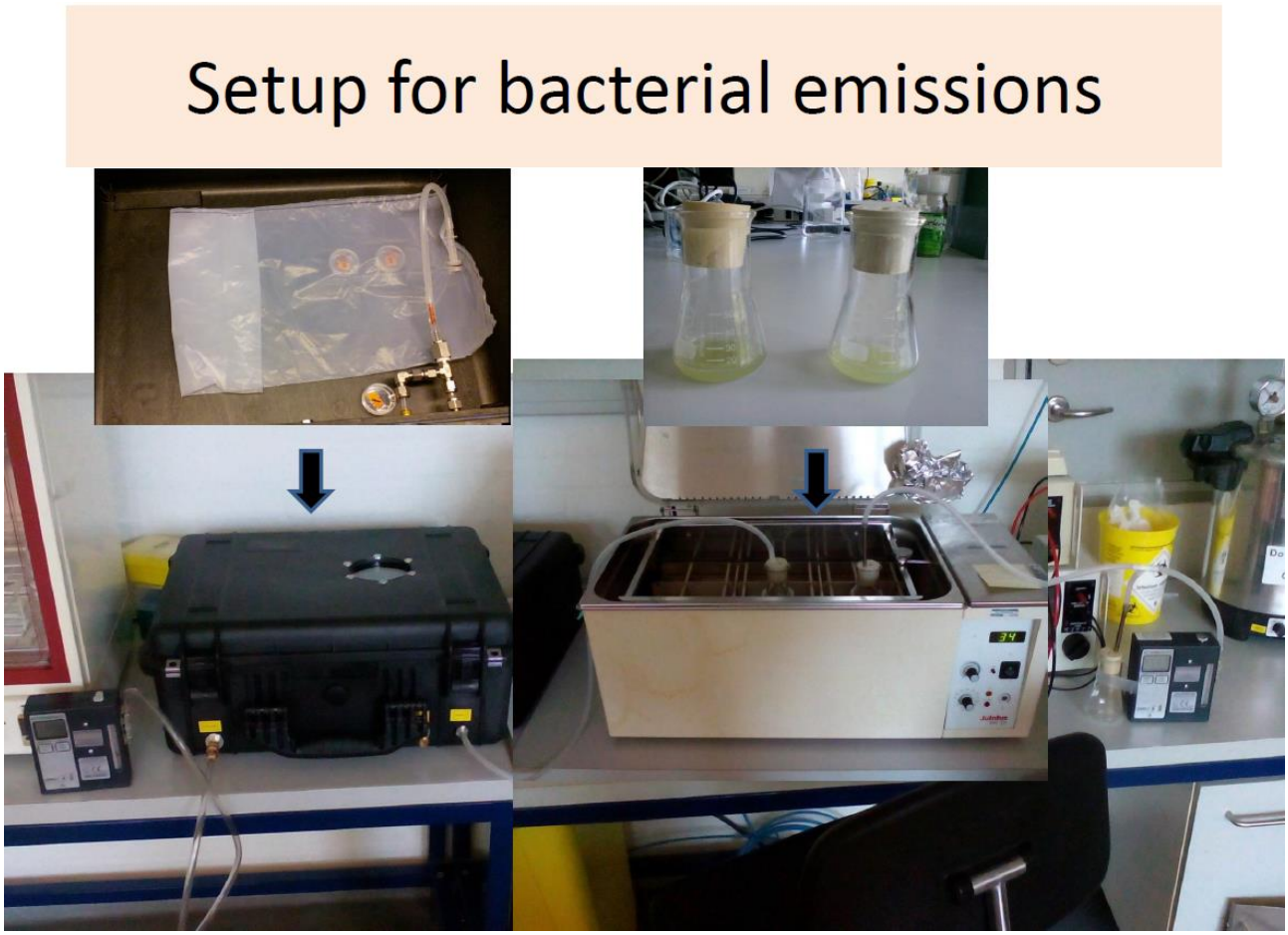
Accidents should be reported to the project manager and to the employees’ safety officer. They should be informed of which culture was involved, and the quantity involved, as well as the time of the accident, and the measures taken to counteract the accident.

The project manager and the employees’ safety officer should note details of accidents in the laboratory log book and, if necessary, report them to the authorities. Introduce necessary precautionary measures to prevent repetition of that type of accident.

11) Signature and Date

Dennis Sandris Nielsen and Marina Bjørklund 08/05-2012

SOP 1: **Cleaning of external equipment.**



In room T-474.

External equipment consists of:

- 2 pumps incl. adapters and extra tools: To be wiped with **70% ethanol**.
- 1 vacuum box: To be wiped (in- and outside) with **70% ethanol**.
- 1 Teflon bag incl. Petri dish with SERS chip inside: Teflon bag is sterilized for 30 min in a **120°C oven**. Closing devices (stick and clamp) are rinsed in **70% ethanol**.
- Rubber plugs: To be rinsed in **70% ethanol**.
- Silicone tubes and connectors: To be rinsed in **15% sodium hypochlorite**.
- ATD tube: To be wiped with **70% ethanol**. Caps for ATD tubes are rinsed in **70% ethanol**.

SOP 2: **Bring external equipment into the lab.**

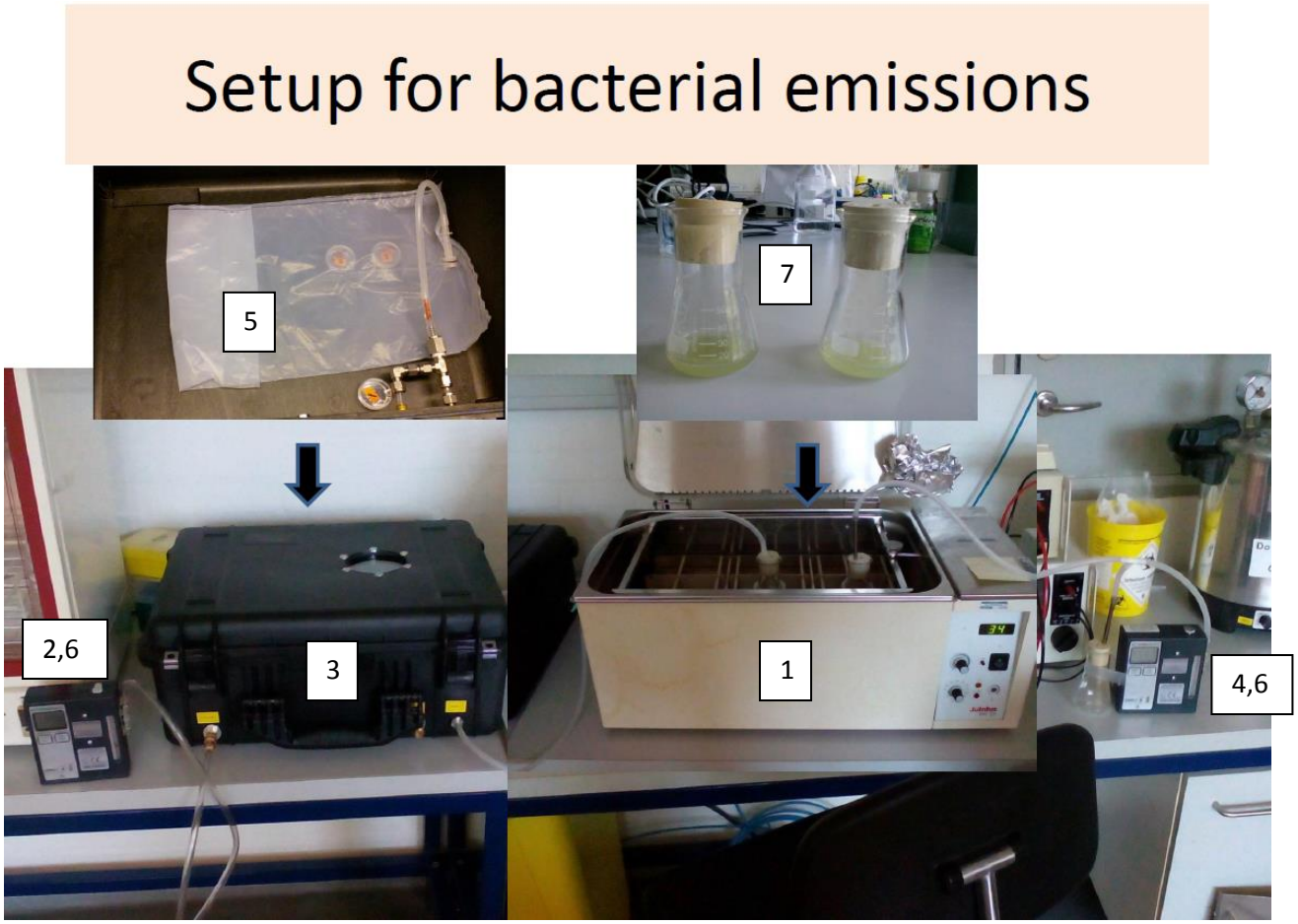
All external equipment (see SOP 1) is brought into the lab on a trolley. Small items such as tubes and connectors are in sealed plastic boxes. If necessary, wipe again with 70% ethanol. E.g. these laminated SOPs.

Before entering a Class 2 lab, put on a lab coat and change it when inside the lab and put on bags to cover the shoes. Make sure the hair is uptight and wear goggles and nitrile gloves.

When leaving the lab, wash the hands and put dirty lab coats in autoclave bags, marked “GMO Kl.2”, which is disinfected on the outside before being sent to the autoclave. Used gloves and shoe bags are thrown into a steel bucket which is then disinfected on the outside with 70% ethanol prior to being brought to the autoclave. At the autoclave, autoclave tape is being put onto the items.

Prior to leaving the corridor use the disinfectant gel on the hands.

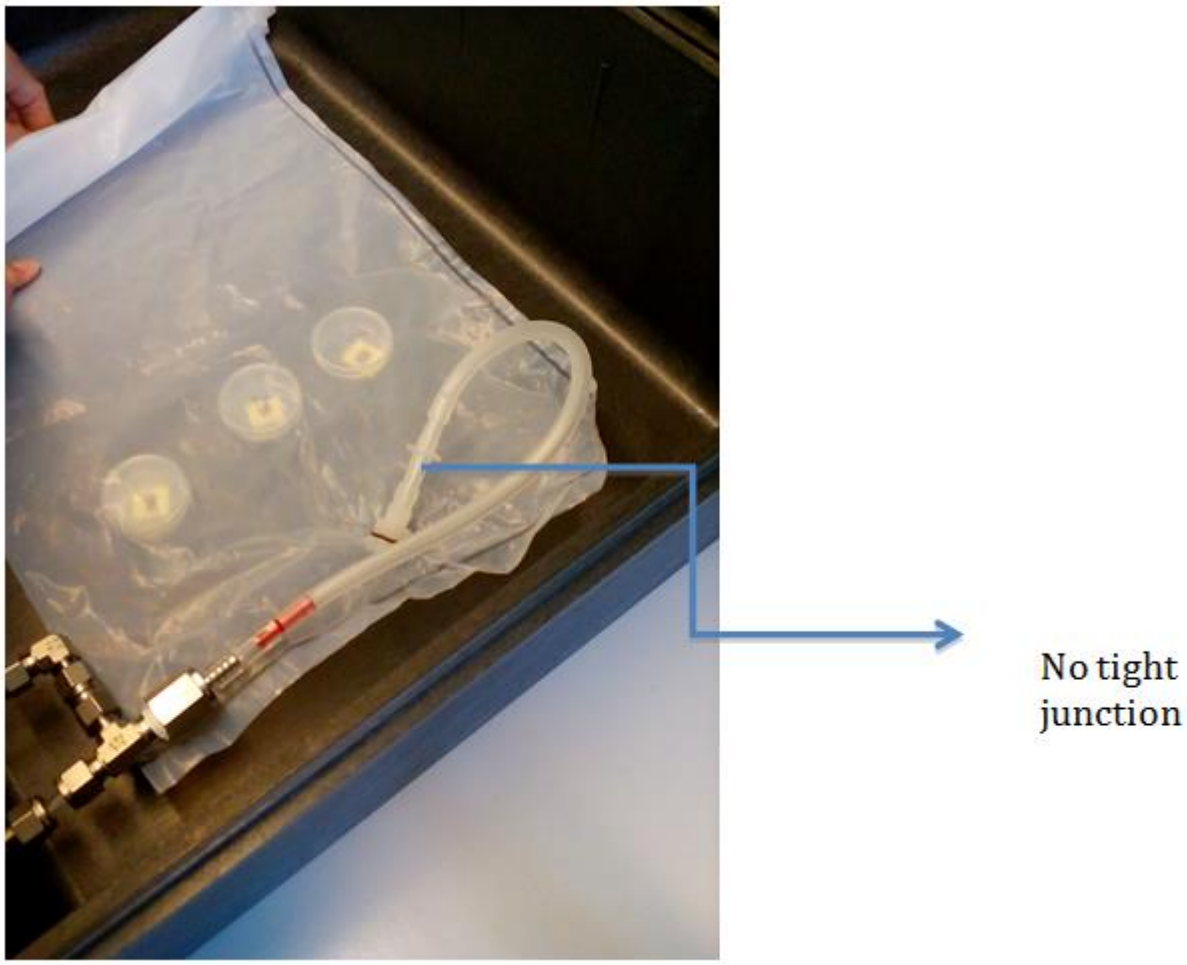
SOP 3: **Set up in- and external equipment.**



In room T-474:

1. A water bath is filled with demineralized water till 1 cm above the bottom of the flasks, and set to 37°C, low shaking.
2. A charged pump is connected to the outlet of the vacuum box via a silicone tube.
3. The vacuum chamber is purged for 20 min.
4. The other charged pump is through a silicone tube connected via a rubber plug and an ATD tube to an empty Erlenmeyer flask (for reference GC-MS). Before this there is a T piece whose other end is connected to another silicone tube which goes on another ATD tube sitting in a rubber plug for the bacterial emission (sample).
5. A SERS chip is mounted in a small Petri dish inside a PTFE (Teflon) bag which is then closed and tightly connected to the inlet of the vacuum box.
6. After each pump a tube is connected which ends in a bath of 15% sodium hypochlorite to avoid eventual aerosol formation. Each pump is also connected to a net adapter for continued function overnight.
7. Two suspensions of PAO1 are prepared. Refer to SOP 5.

SOP 4: **Check the system for leakage.**



As there can be problems with this junction, prior to exposure it should be ensured that it is tight.

SOP 5: **Preparation of PAO1 wet cultures.**

DTU recipe LB broth (from Lisser):

>> 4 g NaCL (merck)
>> 5 g Yeast extract granulated (merck)
>> 10 g Peptone from casein (merck)
>> Ad 1000 ml H2O (orange vand her på DTU) pH 7,40 (2 N NaOH)

LB broth*:	Room:
10 g/L Bacto Tryptone	R-476
5 g/L Bacto Yeast Extract	R-476
10 g/L NaCl	C-423
MQ water (800, then 200 mL)	C-420
ca. 2mL 1M NaOH -> pH 7.2-7.5	A-413

To be **autoclaved before 12.30** in Room C-421 (lid ½ round off) and stored in the fridge.

Bring to the basement:
2 tubes with LB broth in a rack
Key
Disposal bag
Sterile inoculation loops

1. Prepare 2x20 mL of LB broth in two centrifugal tubes and bring them to the basement along with inoculation loops and a disposal bag. **Remember key** for the elevator. For transportation use an appropriate tube rack.
2. **In room T-976:** Open the bag and freezer. Use sterile disposable inoculation loops for harvesting the PAO1 culture from the -80°C freezer 1 (Rack PSN ZDU) into the centrifugal tubes and discard in closed bag after use. Remember to write in the logbook.
3. Vortex the centrifugal tubes before inoculation. Alternatively use shaking in the water bath.
4. A sign saying "We work with *Pseudomonas aeruginosa*" is placed on the lab. door.
5. In **room T-474:** Pour the bacterial suspensions into small Erlenmeyer flasks which are then plugged with double-holed rubber plugs and placed in the 37°C water bath.
6. The centrifugal tubes are put into a sealed (by knot or autoclave tape) autoclave bag in a steel bucket (from entrance of room C-413) which is brought to the autoclave room (**Room C-421**) and marked with black on 4x autoclave tape saying "**PAO1, LB broth, Rikke KL, Lab. T-474**".

*For APVs of the chemicals used, refer to Kemibrug on the pc in the chemical room C-423.

SOP 6: **Mount the system incl. SERS chips and ATD tubes.**

1. SERS chips are mounted inside the 2 small Petri dishes using double-sided adhesive tape with a Post-It on top for repeated light tack.
2. One Petri dish is mounted inside the PTFE bag using double-sided adhesive tape, while the other (reference) is placed on the table.
3. The PTFE bag is connected to the sample opening of the vacuum chamber.
4. The lid is removed from the Petri dish inside the bag which is then closed using the stick and clamp.
5. The vacuum chamber is closed and connected to a bacterial sample in the Erlenmeyer flask via a silicone tube mounted in one hole of the rubber plug.
6. The caps are removed from the ATD tubes and kept for later use.
7. One ATD tube is placed in the rubber plug of the Erlenmeyer flask sitting on the table and connected to the pump via a T piece and silicone tubes.
8. The other ATD tube is placed in the rubber plug of one Erlenmeyer flask containing the other bacterial sample and connected to the T piece via silicone tube.
9. The lid of the other Petri dish is removed.

SOP 7: **Start and run the system.**

1. The 37°C water bath is started to shake carefully (**XXX** rpm), making sure no spillage occurs.
2. The water bath is covered with aluminum foil to prevent heat and water loss or light exposure of the samples.
3. The pumps are turned on. Remember to keep each pump connected to a net adapter for continued use.
4. Check that the flow is about 2 mL/min for each pump. If necessary, adjust the flow using the small screwdriver accompanying the pump.
5. The system is left for overnight exposure, with a sign warning about pathogenic bacteria.

SOP 8: **Stop, secure samples, measure OD and clean.**

1. Shaking and heating is turned off the water bath.
2. The pumps are turned off and disconnected from the tubes and net adapters.
3. The ATD tubes are dismounted and closed by their caps.
4. A lid is put on the Petri dish on the table and sealed with parafilm.
5. The vacuum box is opened, and the PTFE bag inside is transported to the fume hood of R-476 where it is opened. A lid is put on the Petri dish inside the bag.
6. The Petri dish is released from the tape inside the bag and sealed with parafilm.
7. OD is measured on both bacterial cultures:
   In room A-419: To measure OD of the PA culture: Remember to make 2 reference cuvettes, one to put in the ref. spot and one in spot no. 1. The cuvette takes 1mL and the culture can be diluted 10 times directly in this with LB broth medium, i.e. 100µL can mixed with 900µL of LB broth after which it is mixed while in the cuvette sucking the solution up and down a few times with the pipette. A reference cuvette is filled with LB broth and measured first in the 600nm spectrophotometer for calibration. If the 10x diluted sample has an OD of 0.14, the original culture's OD was 1.4.
8. For cleaning: Refer to SOPs 1 and 2.
9. Wearing goggles and a glove the Na hypochlorite flask is taken to the fume hood of **R-476** where it is emptied into the X waste from the locked cabinet in the chemical room, rinsed with distilled water and once more emptied into the waste which is then put back into the locked cabinet. The flask is left inside the fume hood until next day.
10. Regarding PAO1 waste handling and autoclaving: The cuvettes, along with the pipette tips, are discarded in a closed bag and put into an autoclave bag which is put in a steel bucket. The bucket is taken to the autoclave room (**Room C-421**) and closed with 4 autoclave tapes, marked with relevant information regarding its contents. The bacterial suspensions are poured into a Bluecap bottle together with the PAO1 waste and handled in the same way, but in a different steel bucket; one for throwing out after autoclaving, and one for glassware. The Erlenmeyer flasks are rinsed with ethanol which is also poured into the Bluecap bottle, and then placed in the same sealed autoclave bag as the other contaminated glassware.

SOP 9: **Preparation of freeze cultures**:

ON culture:

- LB broth
- 10 mL pipette + tip
- Sterile glass tube in a rack
- Bunsen burner + lighter
- Petri dish with colonies
- Sterile inoculation loops
- "Lab. dancer"

Freeze culture:

- ON culture
- 50% glycerol solution
- 5 mL pipette + 1 mL pipette + tips
- Sterile plastic tube from drawer
- 5x 1 mL Cryo tubes + rack
- **Key**
- Plastic bag

First prepare 37°C ON cultures:

In Room R-476:

1. Fill 10 mL LB broth into a sterile glass tube (sterilize over Bunsen burner before + after filling).
2. Make a small scrape from the Petri dish and put into the LB medium (sterilize before + after).
3. Vortex on the lab. dancer.
4. Put into rack and place in 37°C incubator in room R-478 **over night**.
5. The Petri dish is put into a closed bag and thrown into the yellow bag.

Then prepare 5x the freeze cultures in 20% glycerol: (20% x 1mL) / 50% = 400µL per 1mL cryo tube.

In Room R-476:

6. Take a sterile plastic tube from 3rd drawer. In this:
7. 2mL 50% glycerol is mixed with 3mL ON culture, quickly sterilize over Bunsen burner before + after.
8. Vortex on the lab. dancer.
9. In 5x 1mL cryo tubes: Pipet 1 mL of the solution.
10. The cryo tubes are placed in a freezer box in Rack **PSN ZDU** of freezer 1 in the basement, Room **T-976**.
11. The plastic tube is placed in a bag with a knot and put into the yellow bag.
12. The glass tube is placed in a rack in the autoclave room with autoclave tape marked: "**PAO1 Waste, Rikke K.L., date**"

Preparation of 50% glycerol: (50% x 50mL) / 85% = 30mL.

In Room C-423 (chemicals room):

13. Pour 30 mL of 85% glycerol solution into a 50mL measurement cylinder.
14. Take it to C-420 and add 20 mL MQ water.
15. Cover with Parafilm and turn for mixing.
16. Pour into a 100 mL Bluecap bottle, **autoclave** and store in the cabinet.

Anmeldelse til de Videnskabsetiske Komitéer

20/10/2015

De Videnskabsetiske Komiteer
for Region Hovedstaden
Regionsgården
Kongens Vænge 2
3400 Hillerød

Lyngby, den 20.10.2015

Anmeldelse til de Videnskabsetiske Komitéer

Alle oplysninger på denne blanket kan blive offentliggjort.

Komité
Primærkomité: De Videnskabsetiske Komiteer for Region Hovedstaden
Sekundærkomitéer:
Anmeldelsesnr.:

A. Forsøgsansvarlig
1. Titel: Overlæge, dr.med.
2. Navn: Kim Gjerum Nielsen
3. Hospital/institution: Rigshospitalet
4. Afdeling/institut Afsnit 5003
5. Vejnavn og nr. Blegdamsvej 9
6. Postnummer/7. by: 2100 København
8. Telefonnr.: 35451353
9. E-mail: kgn@dadlnet.dk

B. Evt. anden kontaktperson
1. Titel: PhD studerende
2. Navn: Rikke Kragh Lauridsen
3. Hospital/institution: Danmarks Tekniske Universitet
4. Afdeling/institut DTU Nanotech, Afd. for Mikro- og Nanoteknologi
5. Vejnavn og nr. Ørsteds Plads, bygning 345B
6. Postnummer/7. by: 2800 Lyngby
8. Telefonnr.: 45256397
9. E-mail: rkla@nanotech.dtu.dk

C. Projektinformation
1. Projekttitel: Påvisning af *Pseudomonas aeruginosa* hos patienter med cystisk fibrose ved hjælp af SERS måling af hydrogencyanid i udåndingsluften
2. Projektets hovedformål: Primære endemål er at undersøge muligheden for at skelne mellem patienter med og uden infektion ved hjælp af VC-analyser af udåndingsluft med en nanochip
3. Sted(er) for projektets gennemførelse:
Hospitalsafdeling
Sygehusklassifikationer:
De Videnskabsetiske Komiteer for Region Hovedstaden:

130132W
, Universitetsinstitut
- Adresse(r) / forsøgsansvarlige Rigshospitalet, BørneUngeKlinikken Opgang 5 Afsnit
5003 Blegdamsvej 9 2100 København Ø Institut for
International Sundhed, Immunologi og Mikrobiologi
Panum Instituttet Blegdamsvej 3 2200 København N
Forsøgsansvarlige: 1) Kim Gjerum Nielsen 2) Helle Krogh Johansen 3) Rikke Kragh Lauridsen
4. Forsøgsgrupper: Myndige habile, Mindreårige
5. Design: Kontrolleret, Åbent studie
6. Forskning i biologisk
materiale:
Ingen
7. Lægemiddelforsøg: Nej
8. Medicinsk udstyr: Nej
9. Projektøkonomi: Fondsstøtte
10. Udbetales der vederlag til
forsøgspersoner:
Nej
11. Projekt iværksættes den: 01-01-2016
12. Projekt afsluttes den: 30-03-2016
13. Forventet antal
forsøgspersoner i DK:
70
14. Heraf raske: 20
15. Læge/sundhedsfagligt
område:
Lungemedicin
16. Sygdommens art/navn: pneumoni, cystisk fibrose
17. ICD10 kode: DJ168, DE84
18. Multistat projekt: Nej

_____
Underskrift

Cand.med, Overlæge Kim Gjerum Nielsen
Rigshospitalet
Afsnit 5003
Blegdamsvej 9
2100 København

FORSØGSPROTOKOL FOR:

”Påvisning af *Pseudomonas aeruginosa* hos patienter med cystisk fibrose ved hjælp af SERS måling af hydrogencyanid i udåndingsluften”

Kim Gjerum Nielsen [1]
Helle Krogh Johansen[2]
Rikke Kragh Lauridsen [3]

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Indhold

FORSØGETS FORMÅL

Baggrund

Patienter med cystisk fibrose (CF) danner tykt, sejt slim i lungerne, som let koloniseres med den mikroaerofile bakterie *Pseudomonas aeruginosa*. Hvis der ikke sættes tilstrækkeligt tidligt ind med

antibiotika, kan denne lungeinfektion blive kronisk, med yderligere nedsættelse af livskvalitet og –længde . Derfor skal patienterne hver måned afgive en prøve med ekspektorat (opspyt), som kan blive analyseret for tilstedeværelsen af *Pseudomonas aeruginosa*. Børn yngre end syvårsalderen kan ikke uprovokeret lave et ekspektorat. De må fastholdes og få en slange ført gennem næsen og ned i svælget til fremkaldelse af en hosterefleks, så prøven kan suges op[4]. Denne skal efterfølgende undersøges og dyrkes i 2-3 dage, før resultatet foreligger og evt. behandling kan sættes ind[5].

Pseudomonas aeruginosa (PA) er en af de få bakterier, der producerer hydrogencyanid[6]. Hydrogencyanid er en giftig gas, som antages at bibringe bakterien en fordel i den økologiske niche, den tilhører, og som velvilligt binder til metaller. Hvis hydrogencyanid kunne detekteres i udåndingsluften hos patienter med cystisk fibrose inficeret med PA i luftvejene, ville der kunne spares tid og pinsler for disse patienter og deres forældre, og rettidig behandling ville kunne iværksættes for at undgå, at infektionen bliver kronisk.

Der er på DTU udviklet et SERS (Surface-Enhanced Raman Scattering) substrat, der kan forstærke Raman-signalet op til 1.000.000 gange[7]. Raman er en spektroskopisk metode, der bl.a. bruges til at måle sporkoncentrationer af uønskede stoffer i fødevarer m.v.[8] Substratets overflade er dækket med et lag af guld, som hjælper til at forstærke signalet. Det er vist, at cyanid let binder sig til denne guldoverflade og, at det kan detekteres ved hjælp af det nævnte substrat[9]. Endvidere har forsøg over kliniske PA isolater givet meget lovende resultater (Data endnu ikke publiseret).

Cyanid indeholder en trippelbinding mellem C- og N-atomerne, hvilket giver anledning til et signal i området 2000-2200cm^{-1}, hvor der kun ses trippelbindinger og såkaldt kumulerede dobbeltbindinger[10] (med 2 dobbeltbindinger til samme atom).

Formål

Formålet med dette forsøg er at teste, om hydrogencyanid kan detekteres i udåndingsluften hos børn med cystisk fibrose, der endnu ikke har en kronisk PA lungeinfektion og sammenligne med udåndingsluften fra lunge-raske, frivillige forsøgspersoner. Der henvises til protokol J.nr.: H-3-2012-059, som blev godkendt i 2012 men ikke gennemført, fordi substratet dengang ikke ansås at være klart.

FORSØGETS METODE

Intermitterende koloniserede CF-patienter i alderen 5-18 år, som behandles og går til regelmæssig klinisk kontrol i Dansk BørneLungeCenter og CF Center København afsnit 5003 på Rigshospitalet tilbydes at deltage i forsøget. Desuden tilbydes alders-svarende lunge-raske personer at deltage som kontroller. Prøver af udåndingsluften opsamles fra alle deltagerne, mens det kun er CF patienterne der leverer sputum til mikrobiologisk dyrkning, som en del af den kliniske kontrol, idet raske ikke anses for at have PA. Efter

[4]http://www.rigshospitalet.dk/menu/AFDELINGER/Juliane+Marie+Centret/Klinikker/BoerneUngeKlinikken/Dansk_Boernelungecenter/Cystisk+Fibrose-center+København/Behandling_og_kontrol.htm#larynxsug
[5] http://www.ssi.dk/Diagnostik/DiagnostiskHaandbog/100-199/101.aspx
[6] Enderby, *et al*. (2009): Pediatr Pulmonol, **44**, 142-147
[7] Schmidt, *et al*. (2012): Adv Mat, **24**, *10*, OP11-18
[8] Thygesen *et al*. (2004): Appl Spec, **58**, *II*, 212-217
[9] Lauridsen, et al. (2015): Sensing and Biosensing Research, **5**, 84-89
[10] Williams & Fleming: Spectroscopic Methods in Organic Chemistry, 5th Ed., The McGraw-Hill Companies, UK, 1995, pp. 35, 44f

databehandling vil resultaterne fra patienter og kontroller blive sammenlignet. Det forventes, at deltagere med *P.aeruginosa* vil have et kraftigere Raman-signal i det nævnte område end deltagere uden *P.aeruginosa*. Herudover kan raske personer også have lave cyanidkoncentrationer i udåndingsluften[11], hvorfor det er relevant at inddrage kontroller. Hydrogencyanid findes tillige i mundhulen efter kaffedrikning samt i tobaksrøg[12], hvorfor kaffedrikning, rygning og passiv rygning i hjemmet i dette studie er eksklusionskriterier.

Før udåndingstesten

Forsøgsdeltagerne udfylder et kortfattet spørgeskema vedrørende alder, køn, infektionsstatus, kaffedrikning, rygning samt passiv rygning.

Selve testen

Udåndingstesten gennemføres blandt CF-patienter samt lunge-raske frivillige på Rigshospitalet. Udåndingsluften opsamles dels fra lungerne, hvor børnene selv puster i en teflonpose; og dels fra næsen, hvor der trækkes ca. 10 mL luft gennem en filter "oliven-probe", der sættes hen til næseboret, og som forbindes til en engangssprøjte. I teflonposen og på sprøjtens stempel er der anbragt en nanochip (SERS substratet), der kan opfange og forstærke cyanid-signalet for disse bakterier. Der skal ikke være nogen form for fysisk kontakt mellem forsøgspersonen og substratet.

Efter udåndingstesten

Samme dag som udåndingstesten bringes SERS-substratet til Københavns Universitet, Science fakultetet, hvor der findes et Raman spektroskop med en 1064nm laser, der er særligt velegnet til dette substrat. Signalet mellem 2000-2200cm^{-1} inspiceres for tilstedeværelse af en top, og dennes størrelse (Højde/areal) beregnes. Substraterne kasseres umiddelbart efter endt måling, hvorved der ikke oprettes nogen form for forskningsbiobank.

Mål

Det primære mål er, at bestemme om det er muligt at differentiere mellem koloniserede og ikke bakterieinficerede CF patienter ved hjælp af SERS-analyse af udåndingsluft opsamlet på denne nanochip. Det sekundære mål er at opnå erfaring med håndtering af instrumentet i forbindelse med målinger på patienter og den videre udvikling.

STATISTISKE OVERVEJELSER

Antallet af forsøgsdeltagere er bestemt ud fra, hvad der synes muligt og rimeligt på det aktuelle udviklingsstadium. Idet der er tale om et eksplorativt studie til bestemmelse af metodens sensitivitet og specificitet, forventes dette at kunne danne basis for powerberegning til senere studier i børnegruppen.

[11] Schmidt, *et al*. (2011): J Breath Res, **5**, *046004*, 1-10
[12] Roemer, *et al.* (2004): Toxicology, **195**, 31-52

FORSØGSPERSONERNE, HERUNDER INKLUSIONS- OG EKSKLUSIONSKRITERIER

Antal og fordeling

70 deltagere, heraf 50 CF-patienter uden kronisk *P.aeruginosa* lungeinfektion samt 20 lunge-raske, frivillige. Så vidt muligt 50% af hvert køn. Alder 5-18 år.

Inklusionskriterier

- 5-18 årige CF patienter, som følges i Dansk BørneLunge Center og CF Center København på Rigshospitalet
- Intermitterende PA koloniseret
- 5-18 årige lunge-raske kontroller

Eksklusionskriterier

- Øvrige strukturelle lungelidelser som f.eks. astma el. PCD
- Kronisk PA inficerede CF patienter
- Antistoffer mod PA (≥2,39 Elisa-enheder)
- Antibiotisk behandling mod PA indenfor de seneste 6 måneder
- Ikke i stand til at puste korrekt
- Graviditet eller amning
- Kaffedrikning samme dag som pusteprøve
- Rygning eller massiv passiv rygning (rygning inden døre i hjemmet)

RISICI, BIVIRKNINGER OG ULEMPER

Der forventes ingen risici, bivirkninger eller ulemper for deltagere i forsøget. Det sikres, at deltagerne ikke kan komme i fysisk kontakt med SERS-substratet, der let kan beskadiges og som ikke bør berøres. Det er kun Investigator, som håndterer substratet, og altid iført nitril-handsker og med pincet. Opsamlingen af prøver af udåndingsluften fra CF patienter og kontroller er en non-invasiv procedure, som kan udføres uden at påføre nogen skade eller ubehag. Oplevelsen af ubehag under opsamlingen af udåndingsluft resulterer i omgående ophør af proceduren. Deltageren, forældre eller deltagerens værge kan til ethvert tidspunkt under forsøget annullere det informerede samtykke.

RESPEKTEN FOR FORSØGSPERSONERNES FYSISKE OG MENTALE INTEGRITET SAMT PRIVATLIVETS FRED

For at sikre, at deltagerne lever op til in- og eksklusionskriterierne (jf. pkt. 4.2 og 4.3), kan det blive nødvendigt at indhente de pågældende oplysninger fra deltagernes journal. Data vil blive registreret på PC under overholdelse af lov om behandling af personoplysninger. Projektet anmeldes til den kontaktperson i Region Hovedstaden, der har kontakt til Datatilsynet.

ØKONOMISKE FORHOLD

DTU Nanotech, afdeling for Mikro- og Nanoteknologi, har ansvaret for alle udgifter i forbindelse med forsøget. Rikke Kragh Lauridsens PhD studie er finansieret af Det Frie Forskningsråds Sapere Aude bevilling "NAPLAS", hvorunder udgifter afholdes løbende af et budget på 2 mio. kr. Novo Nordisk fonden har bevilget Helle Krogh Johansens forskningsstipendium. Ingen af de projektansvarlige har nogen økonomisk interesse i projektet. Det er de projektansvarlige, som har taget initiativet til projektet.

VEDERLAG ELLER ANDRE YDELSER

Der ydes intet vederlag eller kompensation for deltagelse. Forsøgets varighed forventes ikke at overstige 15 minutter per deltager, alt inklusive.

HVERVNING SAMT FORLØB FOR INDHENTELSE AF INFORMERET SAMTYKKE

For patienter: Der gives en mundtlig information af overlægerne Kim G. Nielsen, Marianne Skov, Frederik Buchvald eller Tanja Pressler når patienterne er på afdelingen til deres rutinemæssige kontrol. Disse læger kender patienterne i forvejen, og det formodes, at de har de pædagogiske forudsætninger for at formidle til børn og, at samtalen i forbindelse med dette besøg kan foregå uforstyrret. Det oplyses, at de har ret til at have en bisidder ved samtalen, hvis de skønner det nødvendigt. Den skriftlige deltagerinformation udleveres og bringes med hjem, hvor de kan gennemlæse den i ro og mag. Hvis der er behov for yderligere oplysninger, kan de kontakte en af de forsøgsansvarlige, før de beslutter sig, om de ønsker at deltage. Hvis de gerne vil, kommer de til test i forbindelse med deres næste kontrol på afdelingen, som foregår en måned senere, hvor der vil være et særligt rum indrettet til testen, og hvor den nærmere instruktion foregår.

Særligt vedr. de 15-17-årige: Der indhentes informeret skriftligt samtykke hos samtlige forsøgsdeltagere eller disses forældre/værger. Idet testen betragtes som sikker og non-invasiv, forventes det at 15-17-årige selv kan afgive informeret samtykke, hvortil der hermed anmodes om dispensation jf. Komitélovens § 9, stk. 1. Der er udarbejdet skriftlig information til unge mellem 15 og 17 år, som vil blive udleveret.

Projektet kan alene gennemføres ved at inddrage 5-18-årige CF-patienter, og projektet har direkte udsigt til at kunne overføre meget store fordele til den patientgruppe, og indebærer minimale risici og gener for deltagerne.

TILGÆNGELIGHEDEN AF OPLYSNINGER

Deltagerne kan altid kontakte en af de projektansvarlige for flere oplysninger:

Klinisk ansvarlig	**Investigator**
Kim G Nielsen	Rikke Kragh Lauridsen
Overlæge, dr. med.	PhD studerende

Leder,
Dansk BørneLunge Center
Pædiatrisk Klinik I
afsnit 5003
Rigshospitalet
Blegdamsvej 9
2100 København Ø
Tlf.nr.: +45 3545 1353
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DTU Nanotech
Ørsteds Plads
Bygning 345B
2800 Lyngby
Tlf.nr.: 4525 6397
Fax: 4588 7762
Email: rkla@nanotech.dtu.dk

OFFENTLIGGØRELSE AF FORSØGSRESULTATER

Positive, såvelsom negative og inkonklusive resultater af dette studie vil blive offentliggjort i et videnskabeligt tidsskrift.

VIDENSKABSETISK REDEGØRELSE

Der er ikke forbundet ubehag eller skader med opsamlingen af prøver fra udåndingsluften. I studiet undersøges en nanochip, som muligvis kan forbedre diagnostikken af luftvejsinfektioner hos CF patienter, hvorved antibiotisk terapi kan startes tidligere, hvilket har en positiv effekt på bevarelsen af lungefunktionen. Dermed kan metoden være med til at øge livskvaliteten og forlænge livet for patienter med CF.

Hvis det skulle vise sig, at den nye metode ikke er tilstrækkeligt følsom, standses afprøvningen efter 5 falske negativer. Det vil sige, at hvis der i 5 tilfælde ikke detekteres cyanid på nanochippen, hvor den samtidige mikrobiologiske dyrkning af sputum viser sig positiv for PA, afbrydes studiet. Der vil til den tid være gjort vigtige erfaringer med patient-device interaktioner samt håndtering af prøverne, som ikke ville kunne opnås på anden vis.

OPLYSNING OM ERSTATNINGS- ELLER GODTGØRELSESORDNINGER

Patienter er i forsøgsmæssig sammenhæng dækket af Rigshospitalets forsikring.

REFERENCELISTE

http://www.rigshospitalet.dk/menu/AFDELINGER/Juliane+Marie+Centret/Klinikker/BoerneUngeKlinikken/Dansk_Boernelungecenter/Cystisk+Fibrose-center+København/Behandling_og_kontrol.htm#larynxsug

http://www.ssi.dk/Diagnostik/DiagnostiskHaandbog/100-199/101.aspx

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Deltagerinformation til forældre eller værger til lunge-syge børn om deltagelse i det videnskabelige forsøg:

Påvisning af Pseudomonas aeruginosa hos patienter med cystisk fibrose ved hjælp af SERS måling af hydrogencyanid i udåndingsluften

Vi vil spørge jer om tilladelse til, at jeres barn deltager i et videnskabeligt forsøg. Nedenfor følger en beskrivelse af forsøget og de undersøgelser, der indgår i det.

Før I beslutter, om I vil lade jeres barn deltage i forsøget, skal I fuldt ud forstå, hvad forsøget går ud på, og hvorfor vi gennemfører forsøget. Vi vil derfor bede jer om at læse denne deltagerinformation grundigt.

I vil blive inviteret til en samtale om forsøget, hvor denne deltagerinformation vil blive uddybet, og hvor I kan stille de spørgsmål, I har om forsøget. I er velkomne til at tage et familiemedlem, en ven eller en bekendt med til samtalen.

Hvis I beslutter jer for at jeres barn skal deltage i forsøget, vil vi bede jer om at underskrive en samtykkeerklæring. Husk, at I har ret til betænkningstid, før I beslutter, om I vil underskrive samtykkeerklæringen.

Deltagelse i forsøget er frivilligt og I kan når som helst og uden at give en grund trække jeres samtykke tilbage. Tilbagetrækning af samtykket vil ikke have nogen konsekvenser for jeres barns videre behandling.

Baggrund for forsøget

Vi vil undersøge en metode, som gør det muligt at diagnosticere luftvejsinfektioner med Pseudomonas aeruginosa (PA) hurtigere og nemmere end med de nuværende metoder. I metoden indgår et apparat - en nanochip - som i udåndingsluften kan registrere den kemiske forbindelse hydrogen cyanid, som dannes af PA. Denne tilgang til diagnosticering af nedre luftvejsinfektioner har potentiale til at gavne flere patientgrupper, men da patienter med lungesygdomme som cystisk fibrose (CF) relativt ofte kommer til klinisk kontrol og hyppigt lider af nedre PA luftvejsinfektioner, er det indlysende at afprøve metoden på CF patienter.

Formål med forsøget

Forsøgets formål er at undersøge den diagnostiske nøjagtighed af en nanochip i diagnosticeringen af nedre samt øvre luftvejsinfektioner. Det vil sige, at vi vil undersøge, hvor god denne nye metode er til at opspore luftvejsinfektioner ift. de kendte metoder. Hermed får vi kendskab til metodens anvendelighed i klinisk sammenhæng fx på hospitalet eller derhjemme. På længere sigt er formålet at skaffe en hurtigere og nemmere metode til opsporing af luftvejsinfektioner, som forhåbentligt muliggør bedre behandling af luftvejsinfektioner i fremtiden.

Plan for forsøget

Forsøget bliver gennemført fra 01/01/2016 til 30/04/2016. Omkring 50 CF patienter og 20 lunge-raske kontroller bliver tilbudt deltagelse i forsøget. Jeres barn og de øvrige deltagere i forsøget skal undersøges, når de alligevel kommer til kontrol på Rigshospitalet, hvor de vil blive bedt om at afgive en puste-prøve og får suget en luftprøve fra næsen. Udåndingsluften analyseres, og ingen prøve opbevares efter at rå-data fra analyserne er gemt på computeren. Efter rutinemæssige kontrol hentes oplysninger om mikrobiologiske dyrkningssvar[13] og sygdomsstatus fra deltagende CF-patienters journaler.

Forsøget foregår på Rigshospitalet afsnit 5003. Deltagelse i forsøget vil vare ca. 15 minutter, hvor der er afsat tid til puste-prøver og udfyldning af et kortfattet spørgeskema om alder, køn, helbredsstatus, brug af kaffe og rygning samt passiv rygning.

Det er vigtigt at deltagerne faster 2 timer før afgivelse af pusteprøverne. Dvs. jeres barn må ikke indtage kaffe, fast føde eller mælkeprodukter 2 timer før pusteprøverne, men de må gerne drikke rent vand.

Før gennemførelsen af forsøget sikres det, at den informerede samtykkeerklæring er underskrevet og, at I samt jeres barn er informeret om hvad der skal ske. Selve forsøget foregår i følgende trin:

1. Barnet puster i en pose, hvori der er anbragt en nanochip.
2. Barnet puster i en ”party-fløjte”, imens der opsamles 10mL luft i en engangssprøjte fra det ene næsebor gennem en ”oliven-probe”, der føres hen til næseboret. På sprøjtens stempel er der anbragt en nanochip til detektion af gasser fra luften.
3. Herefter skal jeres barn gennemgå den almindelige kliniske undersøgelse, herunder afgivelse af ekspektorat, der bruges til at sammenligne med resultater fra nanochips’ne.
4. De 2 nanochips transporteres til Københavns Universitet, hvor de måles og undersøges for en særlig top (cyanid), der stammer fra PA.

[13] Laboratorie-undersøgelser af opspyt fra lungerne.

Nytte ved forsøget
Det er meget eftertragtet at kunne udføre og tilbyde denne metode til diagnosticering af PA infektioner, da det er en hurtigere, mindre invasiv og mere følsom metode end de gængse. Derfor er der både en økonomisk såvel som en klinisk gevinst i beherskelse af denne metode til diagnosticering af PA luftvejsinfektioner. Hvis resultaterne af denne kliniske afprøvning er lovende, så har patienterne en metode, som kan gøre de kliniske kontroller lettere og muliggør en hurtigere og mere sensitiv diagnostisk af PA luftvejsinfektioner. Hurtigere diagnostik medfører hurtigere behandling, som er med til at forhindre udviklingen af kroniske infektioner. Dette kan på længere sigt være med til at bevare lungefunktionen og dermed forbedre livskvaliteten samt øge den forventede levetid for CF patienter.

Ubehag, risici og komplikationer
Der forventes ingen komplikationer ved deltagelse i forsøget. Det eneste jeres barn kommer i kontakt med er mundstykket, hvor der skal åndes igennem, og en "vat-tut", der sættes hen til næseboret.
Der kan være risici ved forsøget, som vi endnu ikke kender. Derfor opfodrer vi jer til at fortælle os, hvis der opstår problemer med jeres barns helbred, mens forsøget står på. Hvis vi opdager bivirkninger, som vi ikke allerede har informeret om, så vil I naturligvis blive orienteret med det samme, og I vil skulle tage stilling til, om jeres barn skal fortsætte i forsøget.

Udelukkelse fra og afbrydelse af forsøg
Hvis der skulle opstå ubehag under opsamlingen af udåndingsluft, så afbrydes forsøget omgående. I dette tilfælde vil vi påminde jer om retten til at trække jeres samtykke tilbage på et hvert tidspunkt uden følger.

Videregivelse og behandling af oplysninger
Jeres samtykke omfatter også adgang til nødvendige oplysninger om jeres barns helbredsforhold, for at sikre overholdelse af protokollens in- og eksklusionskriterier.

Oplysninger om økonomiske forhold
Forsøgsansvarlige og de tilknyttede videnskabelige medlemmer har taget initiativ til forsøget. Det Frie Forskningsråd støtter Sapere Aude projektet "NAPLAS", som Rikke Kragh Lauridsens PhD projekt er en del af. Novo Nordisk fonden har bevilget Helle Krogh Johansens forskningsstipendium.

Adgang til forsøgsresultater
Negative, positive såvel som foreløbige resultater fra forsøget vil blive offentliggjort. Forsøget forventes at være afsluttet i juli 2016, hvor resultaterne af forsøget vil blive offentliggjort i en videnskabelig artikel.

Vi håber, at I med denne information har fået tilstrækkeligt indblik i, hvad det vil sige at deltage i forsøget, og at I føler jer rustet til at tage beslutningen om jeres barns eventuelle deltagelse. Vi

beder jer også om at læse det vedlagte materiale "Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt" udfærdiget af Den Nationale Videnskabsetiske Komité.

Hvis I vil vide mere om forsøget, er I meget velkomne til at kontakte overlæge, dr. med. Kim Gjerum Nielsen, Rigshospitalet, afsnit 5003, kgn@dadlnet.dk, 35 45 50 06. Rigshospitalets adresse er: Blegdamsvej 9, 2100 København Ø.

Med venlig hilsen

Overlæge Kim Gjerum Nielsen, overlæge Helle Krogh Johansen og PhD studerende Rikke Kragh Lauridsen.

Informeret samtykke vedrørende deltagelse i det sundhedsvidenskabelige forskningsprojekt:

Påvisning af Pseudomonas aeruginosa hos patienter med cystisk fibrose ved hjælp af SERS måling af hydrogencyanid i udåndingsluften

Erklæring fra forsøgspersonen:

Jeg har fået skriftlig og mundtlig information, og jeg ved nok om formål, metode, fordele og bivirkninger til at give mit samtykke.

Jeg ved, at det er frivilligt at deltage, og at jeg altid kan trække mit samtykke tilbage uden, at jeg mister mine nuværende eller fremtidige rettigheder til behandling. Jeg har endvidere modtaget og er blevet opfordret til at læse "Før du beslutter dig" og "Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt" udfærdiget af Den Centrale Videnskabsetiske Komité.

Jeg giver mit samtykke til at deltage i forskningsprojektet og har fået en kopi af dette samtykkeark til eget brug.

Forsøgspersonens navn:

_____.

Dato: _____ Underskrift:

_____

Ønsker du at blive informeret om forskningsprojektets resultater samt eventuelle konsekvenser for dig?:

Ja _____ (sæt X) Nej _____ (sæt X)

Hvis ja, så udfyld venligst en af de følgende:

Telefonnummer: _____

E-mail adresse: _____

Erklæring fra den, der afgiver information:

Jeg erklærer, at forsøgspersonen har modtaget mundtlig og skriftlig information om forsøget. Efter min overbevisning er der givet tilstrækkelig information til, at der kan træffes beslutning om deltagelse i forsøget. Indehaveren af forældremyndigheden til unge deltagere mellem 15-17 år har modtaget den samme information som den unge og er inddraget i den unges stillingtagen til deltagelse i projektet.

Navn på den, der har afgivet information:

_____

Dato: _____ Underskrift:

_____

Spørgeskema til deltagere i forsøget:

Påvisning af Pseudomonas aeruginosa hos patienter med cystisk fibrose ved hjælp af SERS måling af hydrogencyanid i udåndingsluften

Udfyld venligst spørgeskemaet med det samme med kuglepen (sæt X):

Alder: _____ år

Køn: Mand ___ Kvinde ___

Helbredstilstand:
Cystisk fibrose: Ja ___ Nej ___

Anden lunge-sygdom: Ja ___ Nej ___ (Hvis ja, hvilken? _____)

Har du oplevet noget af følgende den sidste uge?

Feber: Ja ___ Nej ___

Hoste: Ja ___ Nej ___

Utilpashed: Ja ___ Nej ___

Brug af kaffe:
Har du drukket kaffe i dag? Ja ___ Nej ___

Hvis ja: Ca. hvad tid? Klokken_____

Rygning:
Ryger du? Ja ___ Nej ___

Udsættes du for passiv rygning i hjemmet? Ja ___ Nej ___

Dato: _____ Navn: _____CPR-nr.: _____

Supplementary

Lyngby, den 31. oktober 2016.

Kære deltagere samt forældre.

Tak, fordi I deltog i den første afprøvning af vores nanosensor til at "fange" gasser fra *Pseudomonas*. Jeg kan fortælle, at der i alt var 50 deltagere med CF og 19 raske kontroller med i forsøget, som løb fra januar til april måned 2016.

Deltagerne blev set alt imellem 1-5 gange i perioden, og der opstod ét nyt tilfælde af *Pseudomonas*, som ikke var hos en i forvejen kronisk inficeret deltager med CF. Det gode var selvfølgelig, at ekstremt få fik *Pseudomonas* i perioden. Omvendt, så giver et enkelt nyt tilfælde mig ikke meget data at arbejde med, og en anden gang vil vi forlænge forsøgsperioden, for at få chancen for at "fange" noget mere. Chippen fra den patient, der fik dyrket *Pseudomonas*, havde et forhøjet signal, mens de øvrige prøver (hvor der ikke blev fundet *Pseudomonas*) fra den samme patient havde et normalt signal.

I løbet af de 4 måneder var der yderligere 2 gange, hvor jeg troede, at jeg havde set spor af *Pseudomonas*, men der blev ikke dyrket noget fra de 2 CF patienters sputum-prøver. Derfor kan det være, at der er mulighed for et "falsk positivt" signal på chippen, og det er selvfølgelig ikke meningen. Da jeg senere fik en ny pusteprøve fra de samme 2 patienter, var signalet igen normalt.

Der var også 2 andre deltagere med CF, hvor man kunne se på pusteprøverne, at der blev røget i deres hjem. Tobaksrøg kan nemlig give det samme signal i udåndingsluften (også hos passive rygere) som *Pseudomonas*. En anden erfaring er, at selve luften i rummet, hvor barnet puster, eventuelt også kan have en betydning. Det er en ganske følsom chip, og det ser altså ud til, at der er en forskel på "baggrunds-luften", der afhænger af hvilket rum patienten har pustet i.

Der var desværre nogle, som havde forstået, at kaffe-drikning kunne give *Pseudomonas*. Jeg vil gerne beklage min formulering i brevet og understrege, at dette IKKE er tilfældet. Kaffe kan give den samme gas i udåndingsluften, som *Pseudomonas* kan; og det var derfor, at deltagerne ikke måtte have drukket kaffe inden de pustede.

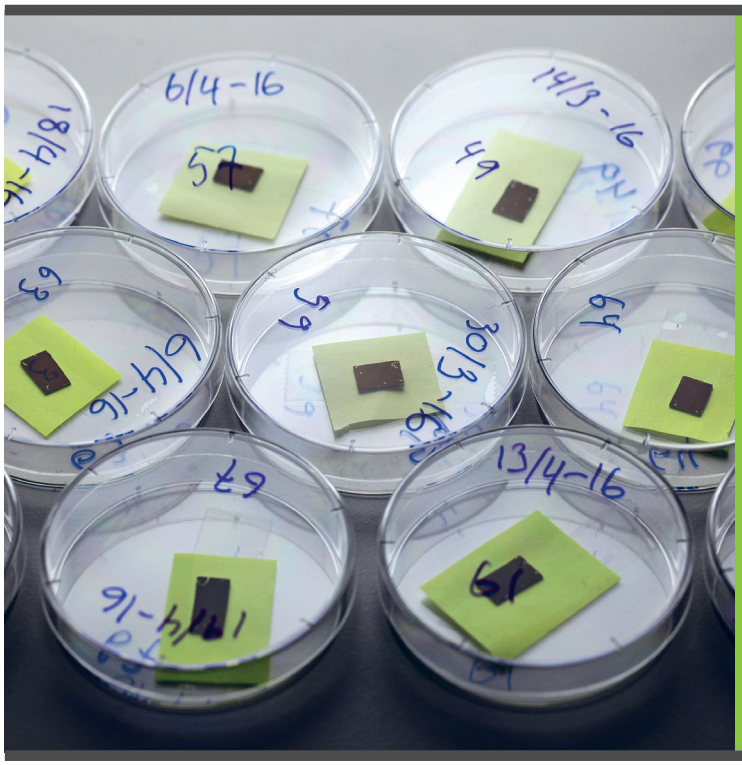
De 4 måneder på Dansk BørneLungeCenter har lært mig, at det ikke er ligegyldigt hvor længe posen får lov til at ligge med chippen indeni. Jeg havde på forhånd besluttet, at den skulle ligge i 15 minutter, men nogle gange fik den lov til at ligge længere, bl.a. fordi rummet, hvor posen skulle tømmes, var optaget.

Der sker nogle gange det, at en sputum-prøve kan være et stykke tid undervejs til mikrobiologerne, som skal dyrke den. Her ser der ud til at være en mulighed for forbedring af metoden til at "fange" *Pseudomonas*, hvis denne tid bliver optimeret.

Jeg skal til at afslutte min PhD, og det er min forhåbning, at der er en anden forsker, som kan overtage projektet, når jeg stopper. Jeg ønsker jer alt det bedste fremover.

Mange hilsner fra

Rikke Kragh Lauridsen.



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